# FACTORS AFFECTING THE EARLY LIFE STAGES OF HATCHERY-REARED GREENBACK FLOUNDER (*Rhombosolea tapirina*, Günther, 1862).

by

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SUMMARY

The major parameters affecting the viability of hatchery-produced eggs, larvae and newly metamorphosed juveniles of the greenback flounder (*Rhombosolea tapirina*, Günther, 1862), were investigated in order to design a suitable method of production.

The production of good quality eggs was considered to be a major problem requiring further investigation. A fertilisation rate of approximately 50% was obtained with most batches of eggs. However, it was found that the broodstock matured normally, even in a completely artificial environment with temperature and photoperiod control. Consistent ovulation of mature eggs was induced artificially, using 'Ovaprim'.

Fertilisation of eggs was carried out successfully both dry and in seawater. A salinity of 35-45‰ was found to be optimal for initial fertilisation with incubation able to be carried out in salinities ranging from 15-45‰ with no effect on subsequent hatch rates. The optimal temperature for incubation of eggs was 9-12°C and incubation at 6 or 21°C resulted in complete mortality. The time to 50% hatch ranged from 1,089 to 1,212 degree hours, depending on the incubation temperature. Fertilised eggs were buoyant in salinities above 27-28‰. At the tail-bud stage eggs could be handled without affecting hatch rates.

The optimal temperature for incubation of larvae at the yolk-sac stage was found to be 15°C, as this temperature resulted in the fastest growth rates and the maximum length at complete yolk absorption. First-feeding occurred at approximately 96 hours post-hatch (day 4) at 15°C, after complete yolk absorption, but before the absorption of the oil droplet. The 'point of no return' occurred at day 6 post-hatch, and mortality of unfed larvae occurred at day 8 post-hatch.

Rearing of the larvae to metamorphosis, was carried out successfully in black fibreglass tanks of 3, 25 and 160 l capacity, contained in recirculating systems and provided with light intensities ranging from 300-1,699 lux. The optimal photoperiod for maximum growth was found to be 18-24 hours light and the optimal temperature was 18-20°C. Culture in salinities of 15‰ resulted in a slight increase in mortality, compared to higher salinities.

Rotifers were found to be a suitable first-feed, with enriched instar II *Artemia* suitable from day 9 post-hatch onwards, when the gape height reached 0.69 mm and the larvae were 4.7 mm in length. The larvae commenced feeding at the water surface but moved to the tank base at approximately day 15 post-hatch at 15°C. At this time they developed a dark colouration. They became lighter at approximately day 20 post-hatch

and developed the juvenile colouration at approximately day 25 post-hatch. Metamorphosis was complete by day 30 post-hatch at a length of 12.4 mm and weight of 25 mg, under optimal conditions. The stomach was fully formed by day 20 post-hatch. Enrichment of live feeds with artificial diets, rather than microalgae, resulted in considerable improvements in the growth rate but a higher incidence of malpigmentation in some trials.

Weaning was carried out successfully from day 23 post-hatch using a 10 day changeover period from live food to artificial food. The weaning diets tested, and the stocking density (between 5-20 individuals/l), had no effect on survival or growth rates. Changeover periods of 10 or 20 days had no significant effect on growth or survival, but a 5 day changeover period resulted in poor growth. Weaning before day 50 post-hatch resulted in the highest survival rates. Larvae showing the highest growth rates prior to weaning were easier to wean, and therefore, larvae fed with live feeds enriched with commercial diets (containing high levels of highly unsaturated fatty acids), showed the best weaning response. Enrichment of live feeds with algae, for the first 15 days of larval rearing, resulted in the maximum post-weaning survival and growth rates.

Growth rates and food conversion rates after weaning were good, and it appears that the most valuable market size (500 g), may be attainable in a 2 year growout period. However, there is a problem with dark pigmentation on the blind sides of most fish and a high incidence of skeletal deformity, possibly caused by a vitamin C deficiency at some stage in the life cycle. Early maturation of 1 year old fish may also be a problem if it results in reduced growth rates.

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**List of common and scientific names of fish species mentioned in the following text.**

| COMMON NAME | SCIENTIFIC NAME |
|---|---|
| Barramundi (Asian sea bass) | *Lates calcarifer* |
| Bay anchovy | *Anchoa mitchilli* |
| Black porgy | *Mylio macrocephalus* |
| Brill | *Scophthalmus rhombus* |
| Cod (Atlantic) | *Gadus morhua* or *callarias* |
| Dab | *Limanda limanda* |
| Dolphin fish (mahi mahi) | *Coryphaena hippurus* |
| Flounder (European) | *Platichthys flesus* |
| Flounder (greenback) | *Rhombosolea tapirina* |
| Flounder (Japanese) | *Paralichthys olivaceus* |
| Flounder (long-snouted) | *Ammotretis rostratus* |
| Flounder (sand) | *Rhombosolea plebia* |
| Grunion | *Leuresthes tenuis* |
| Haddock | *Melanogrammus aeglifinus* |
| Halibut (Atlantic) | *Hippoglossus hippoglossus* |
| Halibut (Pacific) | *Hippoglossus stenolepis* |
| Herring | *Clupea harengus* |
| Milkfish | *Chanos chanos* |
| Plaice (European) | *Pleuronectes platessa* |
| Plaice (Japanese) | *Limanda yokahamae* |
| Rabbitfish | *Siganus guttatus* |
| Rainbow trout | *Oncorhynchus mykiss* |
| Red drum | *Sciaenops ocellatus* |
| Red sea bream (snapper) | *Pagrus auratus* |
| Salmon (Atlantic) | *Salmo salar* |
| Scaled sardine | *Harengus pensacolae* |
| Sea bass | *Dicentrarchus labrax* |
| Sea bream (European) | *Sparus auratus* |
| Sea bream (Western Atlantic) | *Archosargus rhomboidalis* |
| Sole (Dover) | *Solea solea* |
| Sole (English) | *Parophrys vetulis* |
| Sole (lemon) | *Microstomus kitt* |
| Sole (lined) | *Achirus lineatus* |
| Sole (petrale) | *Eopsetta jordani* |
| Spotted seatrout | *Cynoscion nebulosus* |
| Striped jack | *Caranx delicatissimus* |
| Striped trumpeter | *Latris lineata* |
| Tautog | *Tautoga onitis* |
| Tiulka | *Clupeonella delicatula delicatula* |
| Turbot | *Scophthalmus maximus* |
| Turbot (Black Sea) | *Scophthalmus maximus maeoticus* |
| Yellowtail | *Seriola quinqueradiata* |

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1. FISHERIES BIOLOGY

The greenback flounder (*Rhombosolea tapirina*, Günther, 1862) is a commercially important and highly regarded food fish, found in southern Australia and New Zealand. It is most abundant in Tasmania, Victoria and New Zealand, but also occurs in New South Wales, South Australia and Western Australia (Last *et al.*, 1983). The geographical range of *R. tapirina* is limited, compared with those of northern hemisphere and tropical flatfish species (Kurth, 1957).

*R. tapirina* is generally an estuarine species throughout its range and is tolerant of wide variations in salinity (Last *et al.*, 1983). However, it has been caught at depths of up to 100 m. Roper (1979) and Crawford (1984b) both showed that *R. tapirina* was abundant on estuarine sandflats in shallow water of around 0-1 m depth. Juveniles occurred in the highest densities from late winter to early summer in these estuaries and were found to be daytime feeders, consuming mainly amphipods, harpacticoid copepods and polychaete worms. *R. tapirina* adults can reach a length of 380 mm and a weight of 600 g, under natural conditions (Last *et al.*, 1983).

Kurth (1957) studied the growth rate of wild *R. tapirina* and found that at an age of III+ (3+ years from nominated birth date, 31st July) they had attained a length of 31-34 cm (400-500 g). Crawford (1984b) found that first maturity occurred at a length of around 20 cm. Both Kurth (1957) and Crawford (1984) showed that the adults were serial spawners with an extended spawning season from June to October. Jenkins (1987) studied the feeding of wild *R. tapirina* larvae and estimated the growth rates using daily rings on the otoliths. Descriptions of the egg and larval stages, and preliminary larval rearing trials, were recorded only by Crawford (1984a; 1986) who considered that *R. tapirina* could have potential for aquaculture, and recommended further investigation.

*R. tapirina* has a number of characteristics which make it suitable as a species for commercial culture: high market acceptability; unpredictability as a species for commercial fishing resulting in fluctuations in the supply of wild fish; and declining catches. The catch in Tasmania consists largely of an incidental by-catch of Danish-seine fishermen (Crawford, 1984b; Kailola *et al.*, 1993). The annual landings of wild flounder in Tasmania have undergone a steady annual decline from 59,343 tonnes in 1988 to 31,985 tonnes in 1992 (Tasmanian Department of Sea Fisheries statistics, 1993). Growth rates of wild fish appear reasonable and the most valuable market size (500 g+) is attained in three years (Kurth, 1957). However, for many cultured species there has been a considerable improvement in the growth rate under artificial conditions, due to the continuous availability of food and the maintenance of optimal

conditions throughout the culture period e.g. red sea bream (*Pagrus auratus*) (Foscarini, 1988; Bell *et al.*, 1991).

## 1.2. MARINE FISH CULTURE

The culture of marine flatfish was reviewed by White and Stickney (1973), Liewes (1984) and Person-Le Ruyet (1990). Since the development of a hatchery method for commercial production of plaice (*Pleuronectes platessa*) (Shelbourne, 1964; 1975), culture methods have been developed for other, more valuable species such as turbot (*Scophthalmus maximus*), Dover sole (*Solea solea*) (Person-Le Ruyet, 1990), halibut (*Hippoglossus hippoglossus*) (Holmefjord and Olsen, 1991) and Japanese flounder (*Paralichthys olivaceus*) (Shepherd and Bromage, 1988).

The production of *S. maximus* is now a commercial reality, with output in 1991 reaching 929 tonnes (FAO, 1993). Production in 1992 was expected to reach 2,200 metric tonnes (Cachelou *et al.*, 1989), although more recent figures are unavailable. The production of juvenile *H. hippoglossus* is also increasing, with 40,000 fry produced in 1990 (Y. Harache, pers. comm.) and 180,000 in 1991 in Norway alone (Van Der Meeren, 1991). The production of *P. olivaceus* reached 8,315 tonnes in 1991 (FAO, 1993). The total world production of flatfish was 9,327 tonnes in 1991 (FAO, 1993). However, the production of flatfish is still in its infancy compared to that of marine round-fish.

A number of marine round-fish species are being produced in commercial quantities from hatchery-reared stock, in both Europe and Japan and these include sea bass (*Dicentrarchus labrax*), sea bream (*Sparus auratus*), red sea bream (*Pagrus auratus*) and yellowtail (*Seriola quinqueradiata*). In 1991 the production of *D. labrax* and *S. auratus* reached 9,294 tonnes and continues to expand rapidly throughout the Mediterranean region (FAO, 1993). In Japan the production of red sea bream (*P. auratus*) reached 59,500 tonnes in 1991 and that of yellowtail (*S. quinqueradiata*) reached 161,077 tonnes (FAO, 1993). The total world production of marine fish was 308,976 tonnes in 1990, 340,475 tonnes in 1991 (FAO, 1993) and is expected to rise to 350,000 tonnes by the year 2000 (Chamberlain, 1993). The barramundi (*Lates calcarifer*) is the only native species of marine round-fish in commercial production in Australia at present. Production of this species reached 151.4 tonnes in 1991-92 (O'Sullivan, 1994). The only flatfish being studied at this stage, is *R. tapirina* and there is no commercial production as yet.

## 1.3. AREAS OF INVESTIGATION

The commercial production of marine fish involves a number of techniques which will have to be developed for *R. tapirina*. Based on studies of marine fish culture in Europe and Japan, the main areas requiring investigation are:

### 1.3.1. Egg production.

The production of good quality eggs is a bottleneck in the development of most commercial culture species at present. The reasons for this are unknown and a combination of factors is probably responsible. A number of theories are presently being tested: the timing of stripping (McEvoy, 1984; Holmefjord, 1991; Norberg *et al.*, 1991); broodstock nutrition (Dhert *et al.*, 1991; Lam, 1991, Lie and Mangor-Jensen, 1993); handling stress (Pickering *et al.*, 1987; Sumpter *et al.*, 1987; Carragher and Pankhurst, 1991; Campbell *et al.*, 1992); and holding conditions (Devauchelle *et al.*, 1987; 1988). Environmental stimuli such as temperature and photoperiod, either alone or in combination, can be used to manipulate the spawning periods of many marine fish in order to achieve year-round production of eggs (Bye, 1990).

### 1.3.2. Egg incubation.

Modern intensive hatchery methods involve incubation of eggs at high densities and the subsequent transfer to a dedicated larval rearing system for yolk absorption and feeding (Barnabé, 1990; Person-Le Ruyet, 1990).

Sub- or supra-optimal incubation conditions can cause: deformities of the jaw and spine (Bolla and Holmefjord, 1988; Lein and Holmefjord, 1993; Reitan *et al.*, 1993); small size (Holliday and Blaxter, 1960), low survival and reduction in viability of the resultant larvae (Holliday, 1988). The rate of yolk absorption and the size of larvae at first-feeding is highly dependent on temperature (Houde, 1974; McGurk, 1984). The literature on the sublethal effects of environmental conditions during incubation of marine fish eggs, was reviewed by Rosenthal and Alderdice (1976).

### 1.3.3. Larval rearing.

The effects of tank design (Jones *et al* ., 1974; Howell, 1979; Devauchelle *et al.*, 1986; Ostrowski, 1989) and environmental variables such as photoperiod (Jones, 1972; Fuchs, 1978; Dowd and Houde, 1980; Tandler and Helps, 1985; Duray and Kohno, 1988), light intensity (Blaxter, 1968; 1969; Barahona-Fernandes, 1979; Kiyono and Hirano, 1981; Ounais-Guschemann, 1989), temperature (Fonds, 1979; Ronzani-

Cerqueira, 1991) and salinity (Holliday and Jones, 1967; Hutchinson and Hawkins, 1990), on larval growth and survival, have been investigated in order to identify optimal hatchery conditions for the larvae to metamorphosis.

The feeding of marine fish larvae was reviewed by Tucker (1992). Larval marine fish still appear to require live feed organisms at first feeding. Seale (1933) discovered that the nauplii of the brine shrimp (*Artemia* sp.) can be used as an initial feed for some species. The use of *Artemia* allows for better control over the quality and quantity of the food offered, compared to using wild-caught zooplankton (Watanabe *et al.*, 1980; Léger *et al.*, 1986; 1987). *Artemia* are now used regularly as a food for marine fish larvae. However, they are only useful for larvae with a large gape and therefore, a smaller organism is required for most species at first-feeding (Shirota, 1970; Tucker, 1992).

The Japanese discovered that the marine rotifer (*Brachionus plicatilis*) is small enough (80-240 μm) for the larvae of most marine fish at first-feeding. It is easily cultured continuously at high densities of up to 600/ml, using either bakers yeast or marine *Chlorella* sp. as food (Ito, 1960). Intensive commercial culture methods for the majority of marine fish species, presently involve the use of rotifers for first-feeding followed by *Artemia* as the gape size of the larvae increases (Barnabé, 1990; Person-Le Ruyet, 1990; Watanabe and Nomura, 1990). When a new fish species is investigated as having potential for aquaculture, it is necessary to establish a protocol for the timing of the changeover to different sized live feeds. This can be related to the development of the main organs of the digestive system (Segner *et al.*, 1993) and the increase in gape size (Shirota, 1970).

The use of enrichment diets for the enhancement of the nutritional quality of live feeds, is considered to be an important area for study. Jones (1970) first documented the beneficial effects of adding marine *Chlorella* sp. to larval rearing tanks and Watanabe *et al.* (1978; 1980) linked the contents of highly unsaturated fatty acids (HUFA's) in the live feeds, to the survival of the fish larvae. Imada *et al.* (1979) developed a supplemented yeast diet for rotifers, containing n-3 HUFA's and used this successfully to increase the fatty acid composition of the live prey. It is now regular practice to 'enrich' live feeds with artificial diets containing high levels of ecosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), before feeding to larval fish (Léger *et al.*, 1987, Sorgeloos and Léger, 1992).

In the Order Pleuronectiformes (flatfish) there is a problem with inconsistent levels of pigmentation in cultured fish. This is thought to be caused by a nutritional deficiency

or deficiencies during the early larval stages, before the pigment cells are fully differentiated (Grønås *et al.*, 1993; Kanazawa, 1993).

Problems with swim-bladder inflation have hampered the development of marine fish production (Chatain and Ounais-Guschemann, 1990). The lack of a functional swim-bladder has been shown to result in spinal deformities and poor growth. Swim-bladder inflation generally occurs at the time of first-feeding and is thought to be impaired by oil films on the water surface, although light, aeration and salinity can also have an effect (Battaglene and Talbot, 1990; 1993). However, being a member of the family Pleuronectidae, *R. tapirina* does not have a swim bladder at any stage of its development (Kyle, 1913; 1921).

### 1.3.4. Weaning to an artificial diet.

Due to the length of the larval stage and the cost of live food production, it is highly desirable to develop a formulated diet for the larval rearing of marine fish. Although, according to Sorgeloos and Léger (1992) this development poses so many technological difficulties that, even though diets will undoubtedly be developed, they may not represent a cost effective replacement for live feeds. However, many authors have published the results of their work towards developing diets as live feed replacements or, more commonly, as effective weaning diets for marine fish, after a period of feeding on live diets. Various feed formulations have been successful, including moist and dry extruded pellets (Bromley, 1977; 1978; Gatesoupe and Luquet, 1981/82; Person-Le Ruyet *et al.*, 1983; Applebaum, 1985; Bromley and Sykes, 1985; Devresse, *et al.*, 1991). It has been shown that the condition of the larvae, prior to weaning, has a significant effect on subsequent weaning success (Bromley and Howell, 1983; Person-Le Ruyet *et al.*, 1993).

### 1.3.5. Ongrowing

In Europe, marine fish are on-grown in both cages and onshore facilities. Although the cost of production is higher, the latter option is considered the better of the two for the Pleuronectiformes, due to their behaviour (Person-Le Ruyet, 1990). Growth rates and feed conversion rates on dry diets, are similar to those obtained with salmonids, although trash fish or natural diets continue to result in higher growth and survival rates (Kirk and Howell, 1972; Deniel, 1976; Kuhlmann *et al.*, 1981; Person-Le Ruyet, 1990).

Early maturation is considered to be a problem in many cultured fish species and is thought to be induced by optimal feeding conditions during the period of

gonadosomatic growth (Policansky, 1983; Thorpe, 1986). A reduction in the growth rate occurs with maturation and this may be counteracted by the use of sterilisation techniques such as triploidy and gynogenesis (Purdom and Lincoln, 1973; Purdom, 1983; Thorgaard, 1986) or a manipulation of the feeding regime (Thorpe *et al.*, 1990).

There are a number of questions which will need to be answered before the potential of *R. tapirina* as a commercial aquaculture species, can be properly assessed. These include:

1. **Is it possible to produce weaned juveniles on a commercial scale?**

   The only study of hatchery techniques was that undertaken by Crawford (1984a), who concluded that intensive production was possible on a laboratory scale but further research was required in this area.

2. **What is the optimal growout procedure?**

   It is not yet known whether this species can be cultured in cages or whether a land-based system must be employed. The optimal stocking density is unknown. The growth rate and age at maturity of cultured fish is still unknown and therefore the cost of production cannot be calculated.

3. **What are the nutritional requirements?**

   The nutritional requirements of larvae, juveniles and adults are still unknown and may require considerable research. This is still a problem with commercially produced flatfish (Person-Le Ruyet, 1990).

4. **Are there potential disease problems?**

   Potential pathogens such as *Trichodina* sp. and *Aeromonas salmonicida*, have been isolated from wild and cultured fish (Mount Pleasant Laboratories, Department of Primary Industries, Tasmania) and have been mentioned as a cause of significant mortalities of cultured flatfish in Europe (Person-Le Ruyet, 1990).

5. **Can an economically viable market price be obtained?**

   There appears to be the potential for high value, domestic and export markets for larger fish, particularly when sold as a live product. However, there has been no

opportunity to carry out an assessment of the market potential or consumer acceptance of a cultured product.

## 1.4. AIMS OF THE PRESENT STUDY

The following study was undertaken to address the first of the questions raised above, and provide some of the information required in order to design a reliable hatchery technique for the production of *R. tapirina* juveniles for ongrowing. The general aims of the study were:

1. To investigate the performance of captive *R. tapirina* broodstock and establish a suitable method of obtaining consistent egg supplies for studies of larval rearing techniques.

2. To investigate different fertilisation techniques, and the effects of salinity and temperature, on eggs and yolk-sac larvae, in order to establish a suitable method for the consistent production of viable larvae for ongrowing.

3. To investigate the effects of photoperiod, temperature, salinity and live feed enrichment on the growth and survival of larvae, in order to establish a suitable culture technique for maximum growth and survival to metamorphosis.

4. To examine the development of the digestive system and increase in gape size in order to establish a feeding protocol that reduces the requirement for rotifers and *Artemia* and therefore, decreases the cost of production.

5. To investigate diets, stocking densities, duration of the changeover period, age, and condition, in terms of their effect on growth and survival during weaning to an artificial diet. This was carried out in order to establish a suitable and consistent weaning method.

6. To examine the growth rates and age at first maturity of cultured *R. tapirina*, in order to obtain some information on the suitability of the species for commercial ongrowing.

# CHAPTER 2

# SPAWNING AND EGG PRODUCTION

## 2.1. INTRODUCTION

The successful culture of any fish species relies on the consistent availability of good quality eggs. Achieving control of egg production is therefore of major importance in the development of a commercial culture method. Egg production generally involves the stimulation of maturation, induction of spawning and fertilisation of eggs.

Techniques for stimulation of maturation and induction of ovulation have been reviewed by Bye (1990) and Donaldson and Hunter (1983). Generally, it is not possible to stimulate initial maturation with artificially administered hormone preparations alone, and environmental cues are required (Billard, 1993). Bye and Htun-Han (1978; 1979) showed that manipulations of the environment, particularly photoperiod and temperature, can be used to control the timing of reproductive processes in order to achieve year-round egg supplies. This subject was reviewed by Bye (1990).

Final maturation and ovulation can either be induced using hormones, administered by injection or implant, or allowed to occur naturally in the holding tank. Eggs and sperm can be stripped from the fish manually and fertilised artificially, or spawning can occur in the holding tank and fertilised eggs can be collected at the tank outlet using a fine mesh sieve (Foscarini, 1988; Barnabé, 1990; Baynes *et al.*, 1993). Luteinising hormone releasing hormones (LHRH) and gonadotropin releasing hormones (GnRH), or their analogues, have been used commonly in recent studies to stimulate ovulation. The use of dopamine antagonists in conjunction with releasing hormones was reviewed by Peter *et al.* (1993) and appears to result in improved egg quality.

Crawford (1984a) described the first attempts to artificially induce spawning in *R. tapirina* using human chorionic gonadotropin (HCG). However, the results were variable and many eggs contained multiple oil-droplets, which is considered to be an indication of poor egg quality in species having a single oil droplet under normal conditions.

Many studies have been undertaken recently to investigate egg quality in cultured broodstock. These studies have concentrated on a number of factors:

1. **The timing of stripping relative to ovulatory rhythms.**

   Regular, rhythmic ovulatory cycles have been shown to occur in both turbot (*Scophthalmus maximus*) and Atlantic halibut (*Hippoglossus hippoglossus*) within each spawning season. If the eggs are not stripped within a few hours of

ovulation they exhibit reduced fertility (McEvoy, 1984; Holmefjord, 1991; Norberg *et al.*, 1991).

**2. Broodstock nutrition.**

The fertility of marine fish eggs is affected by the nutritional composition of broodstock diets and the feed rate (Watanabe, 1985; Dhert *et al.*, 1991; Lam, 1991; Lie and Mangor-Jensen, 1993). However, Bromage *et al.* (1992) showed that this is not true for salmonids.

**3. Handling stress.**

The stress caused by handling broodstock during gametogenesis and prior to stripping have been shown to cause a reduction in the fertility of eggs (Pickering *et al.*, 1987; Sumpter *et al.*, 1987; Carragher and Pankhurst, 1991; Campbell *et al.*, 1992).

**4. Holding conditions.**

Broodstock holding tanks of low volume and the use of artificial, rather than natural, light conditions, have both been shown to reduce the fertility of marine fish eggs (Devauchelle *et al.*, 1987; 1988).

Artificial fertilisation of marine fish eggs has been described by many authors and both the 'dry' and 'wet' methods have been used successfully to achieve fertilisation (White and Stickney, 1973; Spectorova *et al.*, 1974; Shelbourne, 1975; Howell, 1979; Crawford, 1984a; Kuronuma and Fukusho, 1984). Lein (1991) showed that exposure to water does not reduce the fertilisation rate of halibut (*H. hippoglossus*) eggs until 20 minutes post stripping. This is considerably longer than is the case for the eggs of other commercially cultured species such as the salmonids (Bromage and Cumaranatunga, 1988).

Pelagic marine fish eggs are generally very small and usually contain one or more oil droplets for buoyancy. They are generally buoyant in salinities greater than 25-28‰ (Fonds, 1979; Liu *et al.*, 1991). The eggs of *R. tapirina* are small compared to other marine fish (Table 2.1.1) and contain multiple oil droplets according to Crawford (1986). The presence of multiple oil droplets in pelagic marine fish eggs is often considered to be a sign of poor quality although in some species it appears to be normal. Ahlstrom and Moser (1980) examined the pelagic eggs of over 500 species of marine fish collected from the wild and found only 15% with multiple oil droplets

under normal conditions, compared to 60% with a single droplet and 25% without an oil droplet.

**Table 2.1.1. Egg diameters and hatching times for *R. tapirina* in comparison with a number of temperate marine fish species.**

| Species | Diameter (mm) | Time to hatch (days) | Temperature (°C) |
|---|---|---|---|
| Greenback flounder (*Rhombosolea tapirina*) [1] | 0.75-0.93 | 3 | 15 |
| Atlantic halibut (*Hippoglossus hippoglossus*) [2] | 3.08 | 18 | 5 |
| Japanese Flounder (*Paralichthys olivaceus*) [3] | 1 | 2 | 16-20 |
| Plaice (*Pleuronectes platessa*) [4] | 1.7-2.2 | 17.5 | 7-11 |
| Sole (*Solea solea*) [5] | 1.0-1.6 | 3-8 | 10-19 |
| Turbot (*Scophthalmus maximus*) [5] | 0.9-1.2 | 3-8 | 13-17 |
| Red sea bream (*Pagrus auratus*) [6] | 0.96 | 2-3 | 16-22 |
| Sea bass (*Dicentrarchus labrax*) [7] | 1.15-1.5 | 5 | 14 |
| Sea bream (*Sparus auratus*) [7] | 0.9-0.96 | 3 | 18-20 |
| Striped Trumpeter (*Latris lineata*) [8] | 1.31 | 8 | 11-15 |

[1] Crawford (1984), [2] Blaxter *et al.* (1983), [3] Kuronuma & Fukusho (1984), [4] Blaxter (1988), [5] Person-Le Ruyet (1990), [6] Foscarini (1988), [7] Barnabé (1990), [8] Searle (1991).

The effect of salinity and temperature on the fertilisation rate of marine teleost eggs, was reviewed by Holliday (1988). Fertilisation rates have been found to vary with salinity, but this is complicated by the fact that the salinity at which the broodstock are held, appears to influence the salinity tolerance range of the eggs. Similarly, the temperature at which the broodstock are held may influence the thermal tolerance of the eggs (Hubbs and Bryan, 1974).

The specific objectives of the present study were to examine:

1. The spawning season of *R. tapirina* under artificial conditions.

2. The effect of a commercial hormone mixture (Ovaprim) containing LHRHa, on egg production by *R. tapirina* broodstock, and subsequent egg quality.

3. The effects of different fertilisation methods, either fertilising eggs in water or without water (the 'wet' and 'dry' methods), on the fertilisation rates of *R. tapirina* eggs.

4. The effect of salinity on the buoyancy and fertilisation rates of *R. tapirina* eggs.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Broodstock maturation and spawning

Broodstock were held in a seawater recirculating system consisting of a 2,000 l, grey fibreglass, subsquare (Rathbun) tank with a central, mesh-covered drain and a 50 mm PVC stand pipe to control the water level and allow rapid flushing of the sump. A settling box, dimensions 90 x 50 x 35 cm, with vertical baffles, was used to remove solids and a Dunlop 'Nova 300' pump raised the water up to the biofilter. The biofilter was of the submerged type, dimensions 60 x 90 x 90 cm, and contained 2 cm gravel mixed with shell grit and oyster shells, in a layer 18 cm thick. Two 20 mm airlifts were added in order to provide recirculation of water within the filter. Filtered water was returned to the tank by gravity. A foam fractionator was incorporated in the system for removal of dissolved organic compounds.

The broodstock holding system was situated in an insulated building with temperature and photoperiod control. These variables were both adjusted throughout the year in order to simulate, as closely as possible, the natural environmental conditions and provide the cues for egg maturation. Temperature ranged from 10°C in June to 16°C in February and photoperiod ranged from 8 hours light in June to 16 hours light in December.

Fish for use as broodstock were collected as juveniles of approximately 70 g, from the River Tamar in 1989 and fed with a moist artificial pellet after initial weaning on chopped mussels (*Mytilus edulis*). At the time of stripping in 1990 these fish had reached 350-400 g. The maximum weight recorded during the study was 840 g. The artificial pellet was prepared by soaking steam-pressed trout pellets (Gibsons Ltd, Cambridge, Tasmania; proximate composition = 38.4% protein; 16% fat; 2.7% fibre) for several hours in freshwater and then adding gelatine (12.5 g/kg pellets) and a vitamin supplement. Once it had solidified, the food was frozen for storage and thawed in small quantities prior to feeding. After the 1990 season the broodstock were fed directly with the dry trout pellets as production of the moist pellet proved time-consuming and the dry pellet was readily accepted by most fish.

In 1992 one cultured male from the 1990 spawning season was found to be mature at a length of 24 cm and weight of 234 g. After testing the sperm for motility, this fish was used to fertilise all eggs obtained in that year. Each year twenty of the largest cultured fish from the previous spawning season were selected and kept as broodstock for future years. This was an attempt to improve the broodstock by selection for fast growth. Care was taken not to fertilise eggs with sperm from the offspring of the same parent.

### 2.2.2. Hormone induction

The broodstock were examined visually for ripeness and were considered to be ready for spawning induction when the gonad was observed to be at stage C, identified by Crawford (1984b) (Appendix 1.5.1.). Spawning was induced using interperitoneal injections of 'Ovaprim' (Syndel Laboratories Inc., Vancouver, BC, Canada). Ovaprim contains [D-Arg$^{6}$, Pro$^{9}$ NEt]-sGnRH and a dopamine antagonist (domperidone) (Peter *et al.*, 1993), and was administered at a total dose of 0.5 ml/kg of fish (manufacturer's specification). The fish were anaesthetised in 40 ppm benzocaine and a 1 ml plastic syringe, graduated to 0.02 ml, was used to administer the hormone. The needle was inserted into the peritoneal cavity just posterior to the pectoral fin, on the ocular side of the fish, and two injections were given separated by 2 or 3 full days (manufacturer's specification). The first injection contained 25% of the total dose and acted as a primer, while the second injection contained 75% and was the main stimulating dose. After the second injection, examinations were made twice daily to test for ripeness. When gentle pressure caused eggs to be freely released, stripping was carried out by squeezing on the dorsal and ventral surfaces simultaneously, using the thumb and forefinger, while gently moving the fingers forwards from the caudal fin area towards the head. In 1991 a few fish ovulated without artificial hormone induction and some spawning behaviour was observed e.g. synchronised swimming (Bromley *et al.*, 1986). However, no fish was observed to spawn naturally in the holding tank until 1994 when a few natural spawnings occurred late in the season.

At first, males were also injected with the hormone but this was abandoned as it appeared to arrest the production of sperm. Stripping of males was carried out using the same method as that used for females.

### 2.2.3. Fertilisation

#### (i) General

Eggs were stripped into a clean, dry plastic bowl and egg quality was assessed by visual examination. According to McEvoy (1984), good quality eggs should be perfectly spherical and transparent, showing clear symmetrical first and second cleavages when fertilised. Poor quality eggs were discarded.

*Determination of egg quantity.*

The mean number of eggs contained in a 1 ml sample, was determined by counting five samples of 1 ml and seven samples of 0.1 ml using a dissecting microscope. Samples of 1 ml were counted initially, but contained more eggs than was anticipated and were difficult to count. Samples of 0.1 ml proved easier to count and were equally accurate. Samples were taken using a 1 ml pipette prior to fertilisation and water addition and the procedure was carried out on only one batch of eggs. The total number of eggs in each subsequent batch was calculated by measuring the total volume, in a measuring cylinder prior to fertilisation, and multiplying by the number of eggs per ml (egg diameter was found to be similar in all batches of eggs stripped at Launceston).

*Fertilisation procedure.*

The eggs were poured gently into a 500 ml Pyrex beaker and approximately 0.1 ml of sperm was added. Thorough mixing was carried out by gently swirling the beaker. In some cases sperm could only be obtained in such small quantities that it was necessary to dilute it slightly in order to achieve thorough mixing of the gametes. Clean seawater of 35‰ was added after a 5 minute period.

In 1990 the eggs were left for a further period of twenty minutes, after which both eggs and water were gently poured into a piece of 150 mm pipe with a 150 µm screen on the base. This screen was placed in a fish box (dimension 40 x 30 x 60 cm) containing clean seawater of 35‰. By pouring clean water through the screen, excess sperm was washed out. Without removing the eggs from the water, a 10 l bucket was slipped under the screen and removed from the fish box. At this stage the eggs were ready for transfer to the incubation unit.

In all future years this complicated procedure was dispensed with and instead, clean seawater of 35‰ was added to the beaker of eggs five minutes after the addition of

sperm. When the viable eggs had collected at the water surface, the water was siphoned out from the base of the beaker using a 5 mm siphon tube. This resulted in the simultaneous removal of any dead or unfertilised eggs at the same time as the water and excess sperm. This washing procedure was repeated twice, after which the eggs were distributed into the larval-rearing tanks.

*Determination of egg quality (fertilisation rate).*

Egg quality was determined for each batch of eggs by measuring the fertilisation rate using the following method. Three samples of eggs from each batch were placed in 25 ml sample bottles, containing clean seawater of 35‰, and incubated at a temperature of 15°C. The number of fertilised and unfertilised eggs were counted at the 4-8 cell stage (approximately 4 hours post fertilisation) and calculated as a percentage of the total number of eggs (a batch of unfertilised eggs was examined in the same manner and contained no developing eggs). A clear relationship between fertility, measured at 3.5 hours post-fertilisation, and subsequent hatch rate was demonstrated by McEvoy (1984) for *S. maximus*.

**(ii) Methods of fertilisation**

The aim of this experiment was to test the fertilisation rates using combinations of 'wet' and 'dry' methods, as follows:

1. Eggs+water/Sperm+water.
2. Eggs+water/Dry sperm.
3. Dry eggs/Sperm+water.
4. Dry eggs/Dry sperm.

For each treatment, 1 ml of unfertilised eggs was placed in a 500 ml beaker. Treatments 1 and 2, were mixed with 1 ml of seawater immediately prior to the addition of sperm. Similarly, for each treatment, 0.1 ml of sperm that had been tested previously for motility, was placed in a petri dish and 1 ml of seawater was added to treatments 1 and 3, just prior to mixing with the eggs. After mixing the sperm with the eggs, the four beakers were left for 10 minutes and 25 ml of clean seawater was added. After a further 5 minute period the beakers were made up to 500 ml with clean seawater and thoroughly mixed. Two 1 ml samples (142.5 ± 21.7 eggs/sample; $\bar{x} \pm$ s.e., n=8 samples) were removed from each beaker with a pipette and placed in labelled 25 ml sample bottles. Each bottle was made up to 20 ml with clean seawater and placed in an incubator at 13°C. An assessment of the fertilisation rate was made using the procedure described in section 2.2.3.(i) above.

### (iii) Effect of salinity

To examine the effect of salinity on the fertilisation rate, the same method was used as in section 2.2.3.(i) above for measuring egg quality but with three replicates (145 ± 14 eggs/replicate; $\bar{x}$ ± s.e., n=12 replicates) fertilised at each of five salinities. The salinities used were 5, 15, 25, 35 and 45‰. Seawater was either diluted with distilled water or concentrated with sodium chloride, to obtain the required salinity.

### (iv) Buoyancy of eggs

The salinity at which newly-fertilised eggs sank, was investigated by placing a batch of newly-fertilised eggs in seawater of 35‰ at 15°C. Distilled water, of the same temperature, was gradually added and thoroughly mixed in until the eggs lost buoyancy. Finally, the salinity was measured after thorough mixing.

## 2.3. RESULTS

### 2.3.1. Broodstock maturation and spawning

Over the period of study, some fish were found to contain ripe eggs at the tertiary yolk stage, in every month from May through to February. It was more difficult to induce ovulation in late spawning fish (November to February) and the fertilisation rates were generally poorer. Maturation and ovulation, with subsequent stripping of ripe eggs, occurred up to three times in each female each season, with approximately six weeks between successive spawnings. However, as hormone injections were used to induce each ovulation it is difficult to draw any conclusions from this.

### 2.3.2. Hormone induction

Hormone induction was successful in most cases, with ovulation occurring 24-48 hours after the second injection of 'Ovaprim'. Ovulation was observed as a swelling of the abdomen in the area of the gut and when this occurred, gentle pressure caused eggs to be freely released. The number of eggs/stripping = 163,000 ± 28,000 ($\bar{x}$ ± s.e.). However, egg quality, as measured by the fertilisation rate, was generally poor (Section 2.3.3.(i)).

Almost all eggs contained a single oil droplet (PLATE 1). It was possible to strip eggs daily from each fish for 2-3 days after ovulation had occurred. However, the last batch was generally of poorer quality, and contained a high proportion of eggs with 2-3 oil

droplets. Eggs from the few natural spawnings occurring in 1994, appeared to be of good quality and developed normally, indicating that natural fertilisation must have occurred successfully in the holding tank.

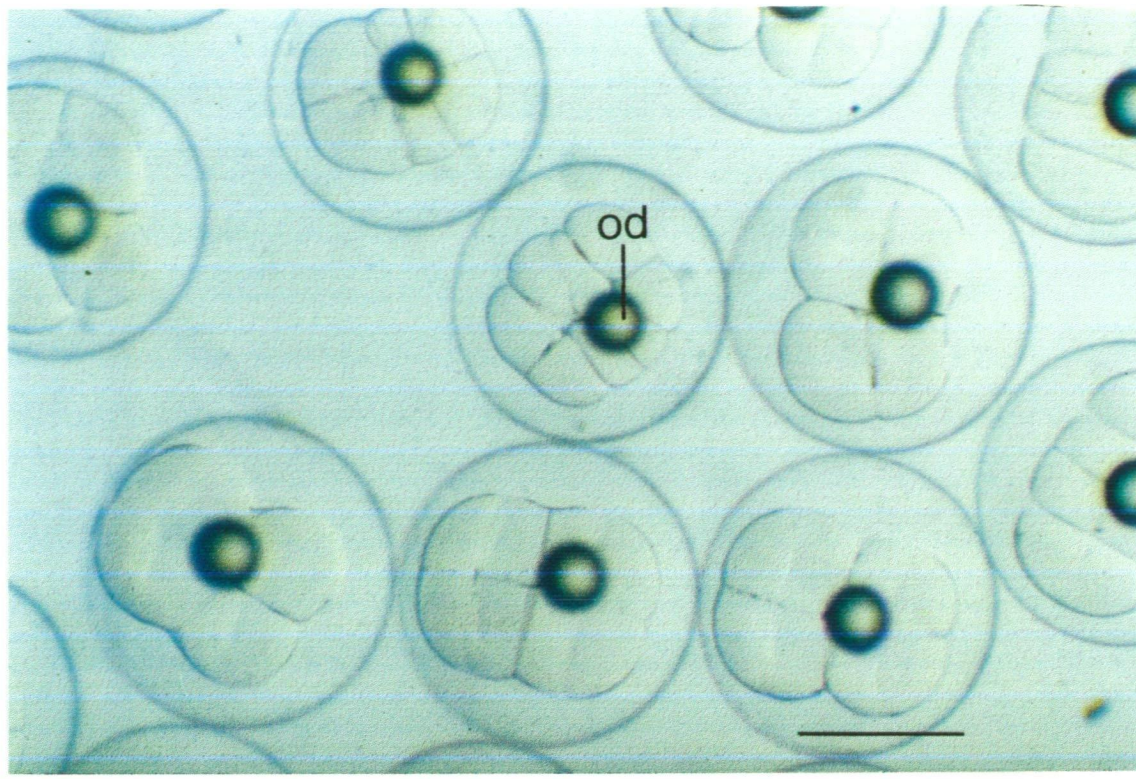


PLATE 1. Eggs of *R. tapirina* at the 4-8 cell stage, showing single oil droplets (od). Scale bar represents 0.5 mm.

Males produced only very small quantities of sperm which was highly active when seawater was added. Activity was observed under a high power microscope and lasted for approximately 2 minutes.

The female genital pore was located on the undersurface of the fish while the male sperm duct was located on the uppersurface and was a combined urinogenital tract. Contamination of sperm with a green substance, presumably urine, was common and may have affected subsequent fertilisation rates of eggs.

The handling associated with spawning induction often resulted in skin damage to the broodstock. Untreated lesions were susceptible to infection by *Flexibacter maritimus*, which often resulted in the death of the infected fish. Treatment with Acriflavine applied directly to the wound was effective in preventing mortality.

### 2.3.3. Fertilisation

#### (i) General

The mean number of eggs/ml or g = 3,504 ± 162 ($\bar{x}$ ± s.e., n=12 samples; measured dry, prior to fertilisation and assuming that eggs have approximately the same specific gravity as water). Unhydrated eggs, just prior to hydration, ranged from 0.37-0.53 mm in diameter. The diameter of hydrated eggs stripped at Launceston, was 0.81 ± 0.02 mm ($\bar{x}$ ± s.e., n=7 batches) while those obtained from the Department of Primary Industry and Fisheries, Taroona, in 1993 measured 0.73 mm diameter, with oil droplets of 0.17 mm diameter and no variation. The mean fertilisation rate at Launceston over the three years was 49 ± 4% ($\bar{x}$ ± s.e., n=10), at 35‰ measured using the technique described in section 2.2.3. (i).

#### (ii) Methods of fertilisation

The addition of water to the gametes, prior to fertilisation, had no effect on the fertilisation rate (Table 2.3.1.). No significant differences (P>0.05) were observed between treatments (Table 2.3.2).

**Table 2.3.1. The effect of fertilisation method on the fertilisation rate of *R. tapirina* eggs ($\bar{x}$ ± s.e., n=2 replicates).**

| Method | Fertilisation rate. (%) |
|---|---|
| 1. Eggs+water/Sperm+water | 56 (4) |
| 2. Eggs+water/Dry sperm | 67 (12) |
| 3. Dry eggs/Sperm+water | 55 (5) |
| 4. Dry Eggs/Dry Sperm | 57 (7) |

**Table 2.3.2. Results of a one-way ANOVA comparing the fertilisation rate (arc sine √ transformed data) of *R. tapirina* eggs fertilised using different methods.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Model | 3 | 0.024 | 0.008 | 0.6443 | **P > 0.05** |
| Error | 4 | 0.049 | 0.012 | | |
| Total | 7 | 0.073 | | | |

### (iii) Effect of salinity

The fertilisation rate of *R. tapirina* eggs was significantly ($P<0.01$) reduced at salinities below 35‰ (Tables 2.3.3., 2.3.4. and Fig. 2.3.1). No fertilisation took place at a salinity of 5‰. Fertilisation rates increased with salinity to a peak at 35-45‰. The fertilisation rate at 45‰ was not significantly different ($P>0.05$) from that at 35‰. The fertilisation rate at 25‰ was significantly higher ($P<0.01$) than at 15‰ but significantly lower ($P<0.01$) than at 35‰ and 45‰.

**Table 2.3.3. Percentage fertilisation of *R. tapirina* eggs fertilised at different salinities ($\bar{x} \pm$ s.e., n=3 replicates).**

| Salinity (‰) | 05 | 15 | 25 | 35 | 45 |
|---|---|---|---|---|---|
| Fertilisation (%) | 0* | 15 (4)$^a$ | 76 (3)$^b$ | 93 (1)$^c$ | 90 (1)$^c$ |

Figures sharing a common superscript are not significantly different ($P>0.05$).

* Not included in statistical analysis.

**Table 2.3.4. Results of a one-way ANOVA comparing the fertilisation rate (arc sine $\sqrt{}$ transformed data) of *R. tapirina* eggs fertilised at different salinities.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Model* | 3 | 1.612 | 0.537 | 170.6 | **$P < 0.01$** |
| Error | 8 | 0.025 | 0.003 | | |
| Total | 11 | 1.638 | | | |

*Data for 5‰ excluded from analysis as variance = 0 and would have interfered with the assumption of ANOVA, that variances should be homogeneous.

### (v) Buoyancy of newly fertilised eggs

At a salinity of 35‰ the eggs of *R. tapirina* floated at the water surface. However, they lost buoyancy at a salinity of 27-28‰.

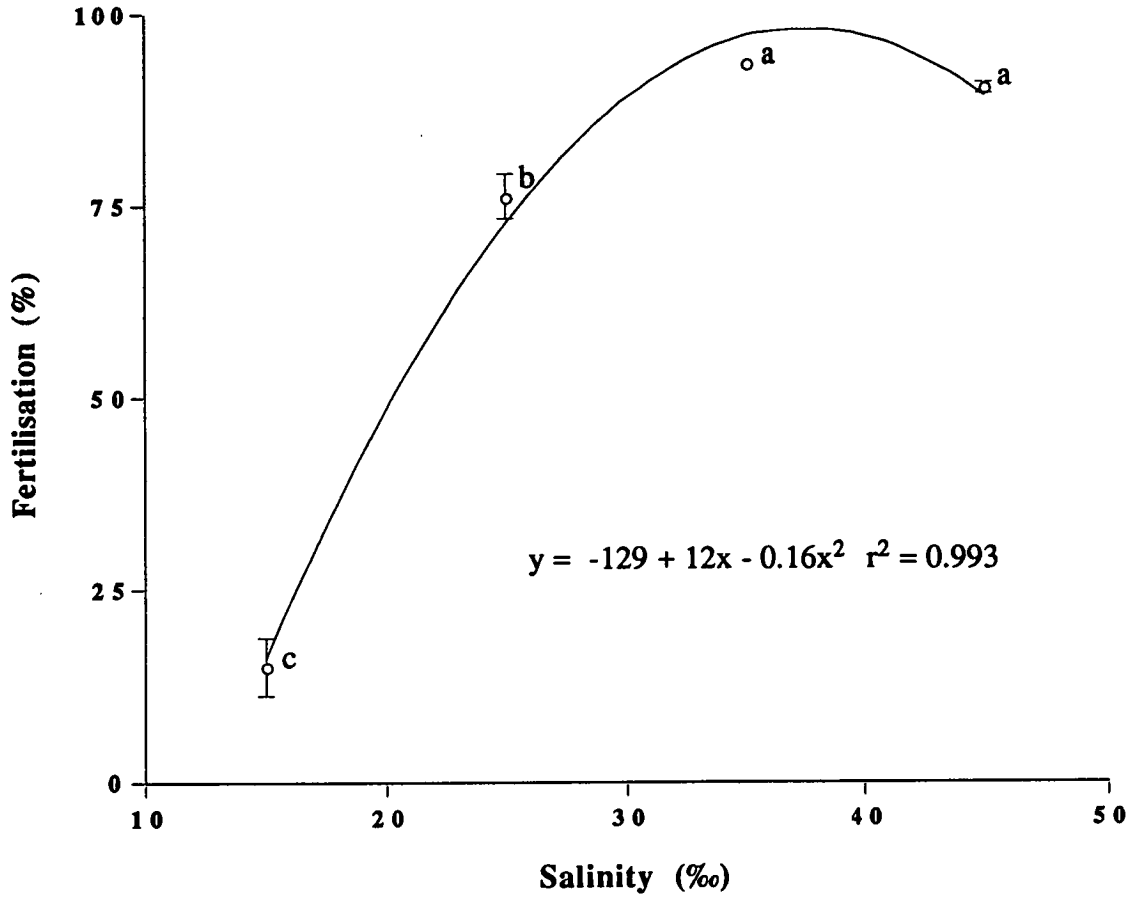


Fig 2.3.1. Fertilisation rate ($\bar{x} \pm$ s.e., n=3 replicates) of *Rhombosolea tapirina* eggs fertilised at different salinities, calculated at 4 hours post-fertilisation.

Points sharing a common superscript are not significantly different (P>0.05).

Points with no visible error bars have very small s.e.

## 2.4. DISCUSSION

*The spawning season*

In the present study, spawning in the greenback flounder (*Rhombosolea tapirina*) occurred with decreasing daylength and temperature in May, through the increasing daylengths in spring, right through to decreasing daylength and temperature in February. However, the majority of successful spawnings occurred between June and October. Both Kurth (1957) and Crawford (1984b) found that spawning of wild *R. tapirina* occurred between June and October and almost all male fish were running ripe between May and October.

Bye (1990) reviewed the literature on the seasonality of spawning in marine teleosts. In this work the main constraints on egg and larval survival are purported to be food availability and predator density. The duration of the daylight period exerts the main influence on phytoplankton production, and hence zooplankton abundance, with temperature as a secondary factor. Maturation and spawning in temperate water fish are generally stimulated by changes in photoperiod and temperature. This ensures that spawning coincides with the period of maximum zooplankton abundance. Photoperiod is a particularly useful stimulus as it does not vary from year to year. Spring spawning is the most common strategy adopted by temperate water marine flatfish species and has been reported by Williams and Caldwell (1978) for English Sole (*Parophrys vetulis*), Shepherd and Bromage (1988) for Japanese flounder (*Paralichthys olivaceous*), and Devauchelle *et al.* (1987; 1988) for turbot (*Scophthalmus maximus*), and Dover sole (*Solea solea*), in the Northern hemisphere.

*Environmental effects on spawning*

The majority of successful spawnings in the present study, occurred in spring, with increasing daylengths from 8-13 hours and temperatures of 10-12°C. However, some fish ovulated with daylengths ranging from 8-16 hours and temperatures of 10-16°C. This shows an extremely wide range of conditions under which maturation and spawning can be induced with this species, although late spawning fish appeared to yield a lower number of eggs and these were of poorer quality. Devauchelle *et al.* (1987; 1988) working with *S. solea* and *S. maximus*, found that spawning occurred with rising temperatures and increasing daylengths in spring, and ceased as a critical temperature was reached. High winter temperatures had a negative affect on subsequent egg production, as did the use of artificial light and temperature control. Egg diameters tended to decrease with increasing daylength and temperature as the spawning season progressed. Bromley *et al.* (1986) found that *S. maximus* produced

small, non-viable eggs at high temperatures. Shepherd and Bromage (1988) reported that *P. olivaceus* also spawn with rising temperatures, and cease spawning as the temperature reaches a maximum level (they did not record the effect of photoperiod).

It appears that temperature is a limiting factor in egg production by the Pleuronectiformes, while photoperiod is the controlling mechanism. *R. tapirina* probably use a similar reproductive strategy, although this was not established in the present study. Critical temperature and photoperiod ranges appear much broader than in other species. Other reproductive strategies are displayed by other groups of fish. For example, reproduction in the red drum (*Sciaenops ocellatus*) can be controlled with temperature alone, while keeping photoperiod constant. This method can result in year-round spawning of a single broodstock (Thomas and Arnold, 1993).

In the present study gametogenesis was successfully induced in *R. tapirina* broodstock contained in a completely enclosed building, by using artificial photoperiod and temperature control. However, as the natural cycles of photoperiod and temperature were followed, it is possible that gametogenesis was controlled by endogenous rhythms. Bye (1990) stated that endogenous rhythms ensure that maturation takes place, and that alterations in environmental conditions can be used to advance or retard the spawning cycle. Therefore, it may be possible to control spawning times in *R. tapirina* using photoperiod, temperature or both, to give year-round egg production.

Bye and Htun-Han (1979) first documented the importance of photoperiod in controlling the timing of reproductive processes. It appears that short days are required to initiate egg development but the timing of spawning can be manipulated using different light regimes. Spawning in *S. maximus* can be controlled by changes in the photoperiod alone with temperature kept constant at 12°C and this allows year-round production of eggs by using three broodstocks kept under different light regimes (Stoss and Røer, 1993). Forés *et al.* (1990) applied a constant photoperiod of 8 hours light per day to broodstock of *S. maximus* and subsequently subjected them to a sudden increase to 16 hours light per day. Most of the females ripened and spawned after 2 months.

*Hormone induction*

Ovulation of *R. tapirina* was successfully induced in the present study, using 0.5 ml Ovaprim/kg body weight, administered over two to three injections. It was not possible to insert a catheter into the genital tract to sample oocytes prior to injection due to the small diameter of the genital pore. The degree of oocyte maturation was assessed by external observation of the ripening ovaries in comparison to the stages

described by Crawford (1984b) (Appendix 1.5.1.). Injections were sometimes unsuccessful, response times varied, and occasionally three injections had to be used. This was probably due to misjudging the stage of maturation.

In the present study, the majority of eggs contained a single oil droplet which probably indicates that the eggs described by Crawford (1986) were of poorer quality. Crawford (1986) used HCG as an inducing agent and may have injected too early, before the oocytes were fully developed. Ramos (1986), was able to induce spawning in *S. solea* using injections of human chorionic gonadotropin (HCG). Low doses were only effective when the oocytes were at an advanced stage of vitellogenesis. Response times varied from 24-96 hours. Crawford (1984a) showed that effective spawning of *R. tapirina*, could be induced with HCG, but the results were very variable. Flüchter (1972b), used the dehydrated pituitary glands of cod (*Gadus callarias*) to induce spawning in *S. maximus* and found that it was only successful if the females had developed a reddish tinge to the genital pore. Egg quality and subsequent survival were very poor. Therefore, it appears that Ovaprim will be a more useful agent for the induction of ovulation in *R. tapirina*, than HCG.

In the present study multiple spawnings were common. This has also been shown in the barramundi (*Lates calcarifer*) after multiple injections of GnRHa (Almendras *et al.*, 1988). These authors showed that a single injection induced a single spawning but two to four injections induced multiple spawnings. However, after using an implant which released GnRHa over a 14 day period, up to five spawnings were recorded. Using a hormone implant had a similar effect on *R. tapirina*, but although viability was high, the eggs contained multiple oil-droplets and were smaller than normal (L. Searle pers. comm.). The number of eggs produced by *R. tapirina* in the present study decreased after 2 days, agreeing with the findings of Almendras *et al.* (1988) for *L. calcarifer* after multiple injections of GnRHa.

*Natural spawning*

Although in the present study natural spawning occurred only in 1994, there were a number of occasions when it may have occurred prior to this, without being detected as the broodstock tank had no egg collector. Spawning behaviour in the form of synchronised swimming was observed on one occasion and occasionally fish that were considered ready to ovulate, were found to have suddenly lost their eggs. Natural spawning of *R. tapirina* was documented by Crawford (1984a), although none of the eggs obtained in this manner were fertilised. In Japan it is not considered necessary to artificially induce spawning and *P. olivaceous* are allowed to spawn naturally in the

broodstock tanks. Spawning occurs morning and evening and continues over a period of approximately 3 months (Shepherd and Bromage, 1988).

Bromley *et al.* (1986) investigated natural spawning of hatchery-reared *S. maximus*, over 4 consecutive years. Males were observed following females and swimming in unison, with synchronous undulations of the body musculature, as observed in *R. tapirina* during the spawning season. Over the four year study period the number of spawnings, yielding viable eggs, increased and the average number of viable eggs released at each spawning increased. No increase in egg production over time was observed in the present study using artificial stripping.

Bromley *et al.* (1986) found that natural spawning resulted in a higher number of viable eggs than hand stripping, and broodstock quality improved with time, either due to ageing, acclimatisation to tank conditions or reduced handling. Devauchelle *et al.* (1987) also obtained better quality eggs from natural spawning than from hand-stripping. Natural spawning would decrease the handling stress associated with injection and stripping of *R. tapirina*. In a study of pilot scale production of *S. maximus* a 10% mortality rate of female broodstock was recorded, due mainly to the stress of hand-stripping (Omnes *et al.*, 1991). Oral administration of hormones would also be effective in reducing stress and labour. Thomas and Boyd (1989) showed this to be an efficient method of inducing spawning of spotted seatrout (*Cynoscion nebulosus*).

*Fertilisation rates*

Fertilisation rates in the present study were very low compared to those achieved by Crawford (1984a). It appears that *R. tapirina* may ovulate during the night and as no stripping took place at this time, the precise time of ovulation may have been missed. Devauchelle *et al.* (1987) found that *S. solea* spawn at night or in the early evening. Crawford (1984a) achieved fertilisation rates of around 85% after stripping during the day. However, this was only achieved with one batch of eggs. The natural fertilisation rate of *S. solea* in the Thames estuary was investigated by Howell *et al.* (1991) and calculated to be in excess of 99%. However, this is rarely possible to achieve under artificial conditions, even if spawning is allowed to occur naturally in the tank (Bromley *et al.*, 1986; Baynes *et al.*, 1993). Many possible explanations for this have been put forward. One of the most recent theories involves the natural ovulatory rhythms that occur in the Pleuronectiformes. McEvoy (1984) showed that each individual female *S. maximus* showed a precise ovulatory rhythm that was always constant. Each female had its own rhythm and therefore, had to be individually marked and stripped as ovulation occurred. The timing of stripping had to be precise

as egg quality deteriorated rapidly if eggs were retained in the ovary after ovulation. This pattern has since been shown for the halibut (*Hippoglossus hippoglossus*) (Holmefjord 1991; Norberg *et al.*, 1991). Fauvel *et al.* (1993) recommended daily stripping of *S. maximus* during the spawning season to alleviate this problem. Ultrasonic scanning was used effectively by Shields *et al.* (1993) to determine the time of ovulation in *H. hippoglossus*. This could be a particularly useful device with *R. tapirina* due to the difficulty of taking catheter samples.

*Broodstock nutrition*

Broodstock nutrition has also been implicated in poor egg quality. Lie and Mangor-Jenson (1993) reviewed the literature on broodstock nutrition and concluded that:

1. There is a shift in the metabolism and redistribution of the nutrient reservoir during maturation.

2. Feeding rates and protein levels in broodstock feeds influence fecundity and the protein source can affect egg quality.

3. The level of essential fatty acids (EFA) has an important effect on egg quality if it is below a threshold level, which is probably species specific.

4. The levels of vitamins C and E are important for securing optimal reproductive performance and larval development.

In the present study, little attention was paid to the improvement of nutrition for maturing broodstock. Feeding levels were probably quite low due to problems associated with high organic loads after heavy feeding in the small recirculation system. Springate *et al.* (1985) showed that if rainbow trout (*Oncorhynchus mykiss*) are fed a reduced ration prior to spawning, the egg size and total fecundity are reduced, mainly by increased rates of atresia of vitellogenic oocytes, but there is no effect on hatch rates or larval survival rates. However, Devauchelle *et al.* (1987) concluded that feeding rates of *S. solea* during the summer and autumn may be an important consideration as the reserves laid down during this period are required to satisfy the increased energy demand required for gametogenesis which occurs during the winter. Starvation of broodstock and nutritional deficiencies have also been implicated in oil droplet fractionation in eggs (Watanabe, 1985; Barbaro *et al.*, 1991). The effect of starvation could explain the small eggs and multiple oil droplets obtained by Crawford (1986) and L. Searle (pers. comm.) at the Department of Sea Fisheries, Taroona.

*R. tapirina* broodstock in the present study were fed with a dry diet formulated for trout. Broodstocks of the main commercial species are commonly fed with either dry diets supplemented with trash fish, or trash fish alone (Foscarini, 1988; Barnabé, 1990; Person-Le Ruyet, 1990). The trash fish is important in ensuring good egg quality. *R. tapirina* will readily take molluscs such as mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*) but the labour requirements are prohibitive. Devauchelle *et al.* (1987) found that an increase in polychaetes in the diet of sole (*Solea solea*) increased the subsequent fertilisation rate of eggs.

*Fertilisation methods*

Fertilisation of *R. tapirina* eggs was achieved using both the 'dry' and the 'wet' method in the present study. Spectorova *et al.* (1974) achieved fertilisation of Black Sea turbot (*S. maximus maeoticus*) eggs by stripping the eggs into a bowl and adding milt from dissected testes strained through muslin cloth. Seawater was slowly added after mixing. However, Lein (1991) showed that the eggs of the halibut (*H. hippoglossus*) remain viable for up to 20 min in seawater so that wet fertilisation is just as effective as dry fertilisation. Liu *et al.* (1991) found that the fertilisation rate of Pacific halibut (*H. stenolepis*) eggs decreased from 50 to 20% between 5 and 10 minutes after the addition of seawater. With *R. tapirina* in the present study, it was found that the quantity of sperm that could be collected from the males by hand-stripping, was very low and it was therefore necessary to mix it with a small quantity of seawater in order to achieve efficient mixing of sperm and eggs. This method has also been used for *H. stenolepis* (Stickney and Liu, 1991).

*Fecundity*

In the present study it was possible to obtain 163,000 eggs/fish at each stripping and this occurred daily for up to three days after ovulation, indicating a potential of around 489,000 eggs/fish. This is low compared to the fecundity of wild *R. tapirina* which was found to range from 820,880 to 1,969,070 eggs/fish for wild fish of 24.7 cm to 34.3 cm or 4,343,000 to 5,250,000 eggs/kg body weight (Crawford, 1984b). This is higher than *P. olivaceous* which produce around 2 million eggs/kg body weight (Kuronuma and Fukusho, 1984). Devauchelle *et al.* (1987; 1988), obtained 140,000 eggs/kg body weight with *S. solea*, and 80,000-200,000 eggs/kg body weight with *S. maximus*, which is considerably less than for the former two species. Egg production in the present study was very low. This may be partly attributable to the serial spawning nature of *R. tapirina*; two or three batches of eggs are shed during each spawning season (Crawford, 1984b). Taking this into account the fecundity of fish in

the present study would be around 1,467,000 eggs/fish (assuming three batches of 489,000/year), which is close to the natural fecundity.

*Egg diameter*

The eggs of *R. tapirina* are very small (0.75-0.93 mm) in comparison with other species (Table 2.1.1) with over 3,000/ml or g. There are 1,800 eggs of *P. olivaceous* to 1 g (Kuronuma and Fukusho, 1984). The petrale sole (*Eopsetta jordani*) has eggs of 1.24 mm in diameter and there are 786 eggs /ml (Alderdice and Forrester, 1968). The small egg size of *R. tapirina* has no disadvantages in commercial culture as the broodfish are small, requiring minimal holding space and have a high fecundity. The larvae have a large gape and feed readily on rotifers, despite their small initial size.

*The effect of salinity on fertilisation rates and buoyancy*

The fact that fertilisation rates obtained in the present study were significantly higher at salinities of 35‰ and 45‰ than at lower salinities, and that eggs were buoyant at salinities above 28‰, indicates that spawning probably occurs at the higher salinities which are encountered offshore. However, the tolerance of eggs to salinity may be affected by the environment of the broodstock (Alderdice, 1988; Holliday, 1988) (Chapter 3). Kurth (1957) believed that spawning occurred inshore while Crawford (1984b) found that ripe males were only encountered offshore during the spawning season (May to October).

Fonds (1979) found that the eggs of *S. solea* lose buoyancy at salinities below 30‰. Liu *et al.* (1991) found that the eggs of *H. stenolepis* change from being neutrally buoyant at 29.8‰ before fertilisation, 31‰ at fertilisation, and 32.5‰ by day 4 post-fertilisation. The salinity of neutral buoyancy then stabilised to 29.8‰ from day 9 post-fertilisation until hatching. For commercial incubation of *R. tapirina* eggs the salinity should be maintained at above 28‰ as it is easier to remove the dead eggs that sink, if the live eggs are floating.

The results of the present study show that:

1. *R. tapirina* broodstock can be stimulated to mature under completely artificial conditions of photoperiod and temperature. Although natural photoperiod and temperature cycles were followed, the opportunity may exist to manipulate environmental conditions in order to achieve year-round production of eggs. Commercial production cycles can be naturally extended due to the long spawning season exhibited by this species.

2. *R. tapirina* broodstock can be induced to spawn using 'Ovaprim' and the quality of eggs appears to be better than those obtained by Crawford (1984) using HCG. The eggs obtained in the present study usually contained a single oil droplet and were more consistent in size, and larger.

3. The eggs of *R. tapirina* produced in captivity, exhibit highly variable fertilisation rates. More research is required to determine the reasons for this in order to improve egg production. Natural spawning techniques should be assessed in order to reduce handling stress and labour requirements during the spawning season; the affects of broodstock nutrition need to be further investigated.

4. The eggs of *R. tapirina* appear to be adapted to a high salinity environment, although this may be due to the broodstock holding conditions. Fertilisation was carried out most successfully in water of 35‰ salinity, using either the 'wet' or 'dry' method and the eggs were buoyant at salinities above 28‰.

# CHAPTER 3

# INCUBATION OF EGGS AND YOLK-SAC LARVAE

## 3.1. INTRODUCTION

In the commercial culture of marine fish, the incubation of eggs is generally carried out in a separate system to that used for larval rearing. Stocking densities are generally higher and either static or slow-flow systems are used depending on the density of eggs. Temperatures are generally lower than those used for larval rearing. Aeration and high salinity water are sometimes required to keep the eggs suspended in the water column (Barnabé, 1990; Person-Le Ruyet, 1990).

The incubation of marine fish eggs demands a thorough knowledge of the effects of important environmental variables such as mechanical stress, salinity and temperature, in order to set incubation conditions which result in optimal hatch rates and viability of the larvae. If the eggs are susceptible to damage from mechanical stress they cannot be disturbed during the incubation period by heavy aeration, high water flows or excessive handling. There is generally low tolerance to mechanical stress up to the closure of the blastopore due to a gradual hardening of the chorion after fertilisation. After this period the eggs become more tolerant to handling stress (Pommeranz, 1974; Kjørsvik and Lønning, 1983; Holmefjord and Bolla, 1988) and can be moved from the incubation tanks to the larval rearing unit (Barnabé, 1990).

Many marine fish species spawn in offshore areas and rely on ocean currents to disperse their progeny. This dispersal mechanism often brings eggs and larvae into areas of varying salinity and temperature, such as estuaries. The effect of salinity and temperature on the incubation of fish eggs, has been reviewed by Rosenthal and Alderdice (1976) and Holliday (1988). Osmoregulation in fish eggs and larvae was reviewed by Alderdice (1988).

The incubation salinity may affect a number of factors such as oxygen availability, specific gravity, ionic concentration, predators, pathogens and osmotic pressures. In marine fish with pelagic eggs a major effect of low salinity is a reduction in buoyancy (Fonds, 1979; Liu *et al.*, 1991). This may have a significant effect on egg survival and the method of incubation used in commercial situations. Sub- or supra-optimal salinities during early development may cause irregular cleavage patterns (Rosenthal and Alderdice, 1976). Holliday and Blaxter (1960) showed that the diameter of herring (*Clupea harengus*) eggs increased with decreasing salinity as did the length and weight of the larvae. Time to hatch was longer in low salinities due to greater regulatory demand. The increased size of the larvae may improve their viability at first-feeding. Larger larvae are able to find food and escape predators more efficiently due to greater strength and swimming speed (Blaxter, 1988).

Sub- or supra-optimal temperatures have been associated with increased levels of spinal and jaw deformities in the larvae at hatching (Bolla and Holmfjord, 1988; Lein and Holmefjord, 1993; Reitan *et al.*, 1993). Deteriorations in water quality are thought to be a cause of spinal deformities in fish larvae (Shelbourne, 1956).

After hatching, there is a short period during which the larvae rely on their yolk for energy and growth. The growth rate during yolk absorption can be reduced by a number of factors including: sub- or supra-optimal conditions of temperature, pH, salinity, light, low dissolved oxygen concentrations, and sublethal concentrations of toxic xenobiotics. The reduction in growth is probably caused by the increased cost of maintenance (Heming and Buddington, 1988). Incubation conditions during this stage must ensure optimal growth rates so that the larvae reach their maximum size before first-feeding.

First-feeding in many marine fish larvae occurs after the absorption of the yolk sac, before the oil droplet has been fully absorbed. This provides for a short period during which the larvae must find food before a critical period is reached, after which the larvae will die even if food becomes available. This critical period is termed the 'point of no return' (PNR) (Blaxter and Hempel, 1966). The time to reach the PNR increases when the eggs are large, when the larvae have a long incubation period and when temperatures are low (Blaxter, 1988).

In many fish the development of the swim bladder occurs at or around the time of first-feeding. Incomplete or abnormal inflation can be a problem in culture and has been the cause of spinal deformities and poor growth in commercially cultured species (Chatain, 1987). The causes have been identified as oil surface films which prevent the fish from gulping air at the water surface (Chatain, 1989, Chatain and Ounais-Guschmann, 1990), light levels in negatively phototactic species preventing the fish reaching the water surface (Battaglene and Talbot, 1990), high aeration and low salinity, probably also affecting the fishes ability to reach the water surface (Battaglene and Talbot, 1993). Oil films can be removed using a surface skimmer (Foscarini, 1988; Chatain, 1989). Among the Pleuronectiformes, both the Bothidae and the Soleidae inflate a swim bladder at first-feeding but lose it after metamorphosis.The Pleuronectidae are unusual as they do not inflate a swim bladder, even during the pelagic larval stages (Kyle, 1913; 1921).

The specific objectives of the present work were to identify:

1 The tolerance to mechanical stress of *R. tapirina* eggs at the tail-bud stage.

2. The optimal conditions of temperature and salinity for incubation of the eggs and yolk-sac larvae of *R. tapirina*.

3. The time of first-feeding in *R. tapirina* larvae and the time to reach the PNR in relation to the absorption of the yolk sac and oil droplet.

*R. tapirina* do not have a swim bladder at any stage in the life cycle and so swim bladder inflation was not studied.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Conditions for incubation

For batches of eggs to be reared through to the larval stages and not studied in the egg stage, incubation was carried out in 160 l black hemispherical, larval-rearing tanks (Section 4.2.1 and Appendix 1.3.). 1990 was an exception in that larval-rearing, and therefore egg incubation, were carried out in 25 l tanks. In that year incubation was attempted in screened containers suspended in the rearing tanks, with the inlet water flowing through the screen. However, it was observed that the eggs were sucked onto the screen by the flow of water and so this was abandoned. In future years the eggs were incubated freely in the larval rearing tanks. In all years the main parameters for egg incubation were similar. Continuous light of 500 lux was supplied by overhead fluorescent lamps, temperature was set at 15°C and a static system or a flow rate of 45 l/hour was used. Aeration was not used in the rearing tanks.

### 3.2.2. The effect of physical disturbance

In order to determine a suitable method for the transfer of eggs from the incubation tanks to the larval rearing tanks, with the least possible mortality, it was necessary to examine the vulnerability of eggs to disturbance at a stage prior to hatching. In this experiment three replicate 25 ml beakers were stocked with eggs at the tail-bud stage, dipped directly from the incubation tank in water, with the minimum of disturbance. A second set of three replicate beakers was each stocked with eggs transported from the incubation tank in a plastic sieve with no water. The mean number of eggs/beaker = $31.83 \pm 4.67$ ($\bar{x} \pm$ s.e., n=6 beakers). The number of larvae hatching in each beaker was recorded.

### 3.2.3. The effect of salinity and temperature

#### (i) The effect of salinity and temperature on eggs

*Experiment 1*

In 1990 an initial experiment was carried out to determine the effect of salinity and temperature on the development and hatch rates of eggs. Each of nine 250 ml beakers was filled with seawater of 15, 25 or 35‰, and fifty eggs were counted into each. One beaker of each salinity was incubated at each of three temperatures, 3-4°C, 13-14°C and 22-23°C. This was carried out by placing the eggs in different areas of the Aquaculture Centre. The developmental stage was determined by removing one to three eggs from each beaker at regular intervals and observing under a low power binocular microscope. The results of this experiment were used as a guide for other incubation experiments. An approximate time to hatch was calculated as well as the temperature tolerance limits for the eggs. The effect of salinity at 13-14°C was measured in terms of survival. The rate of deformities at hatching, such as curvature of the notochord or elongation of the lower jaw, was also recorded as a percentage of the hatched larvae.

*Experiment 2*

In 1993 a set of five water baths were obtained, two of which were both heating and cooling, while the other three were heating only. This enabled five temperatures to be investigated if the hatchery temperature was set at 15°C.

Three preliminary trials were carried out in the water baths in order to test the experimental method. A number of potentially limiting factors were identified and these had to be overcome before a final experimental procedure could be defined. The experimental design considerations are described in Appendix 1.6.

*Final experimental design*

In order to investigate the effect of salinity and temperature on the eggs of *R. tapirina* a multifactorial experiment was undertaken using four salinities and five temperatures. A small number of eggs (39 ± 2, 195 eggs /l; $\bar{x}$ ± s.e., n=60) at less than 1 hour post-fertilisation, were added to each of three replicate 200 ml beakers containing artificial seawater (Forty Fathom, Bio-Crystals Marinemix, from Marine Enterprises, Inc, Baltimore, U.S.A. ) at either 15, 25, 35 or 45‰. Three replicate beakers at each salinity were placed in each of five water baths set at either 6, 9, 12, 15 or 18°C (a

total of 60 beakers). Lids were placed loosely on the beakers to prevent evaporation. The effect was measured by the hatch rate. Hatched larvae were counted hourly during the hatching period and the time at which 50% of the larvae had hatched, was calculated and expressed in degree hours (°h) i.e. the temperature (°C) x time (h).

**(ii) The effect of temperature on the growth of yolk-sac larvae.**

Three preliminary trials were carried out with yolk-sac larvae in the water baths in order to test the experimental method. A number of potentially limiting factors were identified and these had to be overcome before a final experimental procedure could be defined. The experimental design considerations are described in Appendix 1.6.

*Final experimental design*

In order to investigate the effect of temperature on the final length, growth rate and survival of larval *R. tapirina* at the time of complete yolk absorption, batches of twenty newly hatched larvae (initial length = 2.31 ± 0.02 mm; $\bar{x}$ ± s.e., n=10 larvae) were counted into 200 ml plastic beakers containing seawater at 15°C, filtered to 1 µm. Three beakers were placed in each of five water baths set at different temperatures. The temperatures used were 9, 12, 15, 16.5 and 18°C. Lids were placed loosely on the beakers to prevent evaporation. A 25% water exchange was carried out every second day, using temperature adjusted seawater, contained in 1 l beakers placed in each water bath. When the yolk and the oil droplet had been absorbed the remaining larvae were counted and measured. Deformities such as curvature of the notochord and misshapen jaws were also recorded as a percentage of the hatched larvae.

Growth rate was calculated using the formula:

$$\text{Growth rate(mm/day)} = \frac{\text{final length (mm) - initial length (mm)}}{\text{time (days)}}$$

This formula assumes linear growth rates for all treatments but was deemed valid by Hopkins (1992) for this type of experiment.

**(iii) The effect of temperature on growth and yolk absorption rates.**

In order to investigate the effect of temperature on the growth and yolk absorption rates of yolk-sac larvae, an unreplicated experiment was carried out in 3 l buckets. One 3 l white bucket containing a batch of newly hatched *R. tapirina* larvae (initial length = 2.08 ± 0.02 mm; $\bar{x}$ ± s.e., n=10 larvae) in 1 µm filtered seawater at 15°C, was placed in each of five water baths adjusted to temperatures of 9, 12, 15, 18 and 21°C.

Water exchanges (25%) and salinity adjustments (to 35‰) were carried out daily. Ten larvae were removed randomly, from each bucket on a daily basis and the notochord length was measured using a microscope with an eyepiece graticule. The height and length of the yolk-sac and the diameter of the oil droplet, were also measured.

Growth rate was calculated using the formula described in Experiment (ii)., above.

Yolk-sac volume was calculated using the formula:

Yolk-sac volume (V, $mm^3$) = $(\pi/6) \times LH^2$ (Blaxter and Hempel, 1966)

Where L=yolk-sac length (mm) and H=yolk-sac height (mm).

### 3.2.4. Timing of first-feeding

In order to investigate the time at which larvae can first ingest and digest live feeds, six batches of yolk-sac larvae were offered food for the first time, on subsequent days after day 3 post-hatch (the day of hatching is designated as day 0 post-hatch in all future references to days post-hatch). Day 3 post-hatch was used as the starting day as larvae had previously been observed to commence feeding at either days 4 or 5 post-hatch at 15°C. A set of 18, black hemispherical tanks of 3 l capacity (Section 4.2.1) were each stocked with fifty flounder larvae at day 2 post-hatch. Tanks were arranged randomly with six treatments of three replicates. Rotifers (*Brachionus plicatilis*) were enriched with microalgae (Appendix 1.2.) and added twice daily at a concentration of 10/ml to treatment 1 from day 3 post-hatch. Four of the five remaining treatments were first-fed on consecutive days up to day 7 post-hatch and the remaining set of tanks were left as unfed controls. All tanks contained static seawater at a temperature of 15-16°C and salinity of 33-35‰ throughout the trial, with continuous light of 300-400 lux provided by overhead fluorescent tubes. Water was exchanged (approximately 25%) on alternate days. Mortalities were monitored daily where possible, though due to their small size and speed of decay many of the early mortalities were not detected. All survivors were measured (notochord length) after the unfed controls had died, at day 12 post-hatch.

## 3.3. RESULTS

### 3.3.1. Conditions for incubation

Incubation in the rearing tanks was generally successful although fungal contamination caused high mortality on one occasion and bacterial contamination caused 100% mortality on two occasions. It appeared that water quality was critical and water filtered to 1 μm was effective in reducing mortality. A low water flow was

more successful than a static system when high densities of eggs were incubated and it was important to restrict stocking densities to a maximum of around 200 eggs/l. High mortalities generally occurred at day 1 post-fertilisation and during the yolk-sac stage.

### 3.3.2. The effect of physical disturbance

Physical disturbance at the tail-bud stage had no significant effect ($P>0.05$) on subsequent hatch rates (Tables 3.3.1. and 3.3.2)

**Table 3.3.1. Hatch rates of *R. tapirina* eggs after being either, transported in water or removed from the water, at the tail-bud stage ($\bar{x} \pm$ s.e., n=3 replicates).**

| Method of transport | Hatch rate (%) |
|---|---|
| With water | 78 (8) |
| Without water | 71 (8) |

**Table 3.3.2. Results of one-way ANOVA comparing the hatch rates (arc sine $\sqrt{}$ transformed data) of *R. tapirina* eggs after being either, transported in water or removed from the water, at the tail-bud stage.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Model | 1 | 0.001 | 0.001 | 0.054 | **P > 0.05** |
| Error | 4 | 0.086 | 0.021 | | |
| Total | 5 | 0.087 | | | |

### 3.3.3. The effect of salinity and temperature

#### (i) Eggs

*Experiment 1*

Eggs developed normally even when non-buoyant. At 13°C and 25‰ hatch rates were not different from 35‰ (Table 3.3.3.). However, at 15‰ the hatch rate was considerably reduced and the number of deformed embryos increased. No eggs hatched at either 3-4°C or 22-23°C and mortality occurred at an earlier developmental stage in combinations of either, low salinity (15‰) and low temperature (3-4°C) or high salinity (35‰) and high temperature (22-23°C). The time to hatch at 13°C was 90 hours or 1,170°h.

**Table 3.3.3. Mean hatch rates and rates of deformity of *R. tapirina* eggs incubated at different salinities at 13°C (n=1 replicate).**

| Salinity (‰) | Hatch Rate (%) | Deformity Rate (%) |
|---|---|---|
| 15 | 81 | 35 |
| 25 | 97 | 3 |
| 35 | 95 | 3 |

*Experiment 2*

Only the hatch rate was calculated in this experiment and there was a significant effect of temperature only, with no significant ($P>0.05$) interaction effect between temperature and salinity (Tables 3.3.4. and 3.3.5). However, there was a trend towards higher hatch rates at combinations of low temperature/low salinity and high temperature/high salinity. This was significant at $P<0.07$ and therefore may be important. There were only five survivors at 18°C and none at 6°C. Salinities of 15-45‰ had no significant effect ($P>0.05$) on hatch rates. The hatch rates of eggs incubated at 15°C were significantly lower ($P<0.05$) than those incubated at either 9 or 12°C, and the highest hatch rate was recorded at 12°C and 35‰.

The time to 50% hatch decreased with temperatures in a linear fashion between 9 and 15°C (Fig 3.3.1.). However, at 18°C the curve becomes less steep with temperature having less effect on hatching times.

**Table 3.3.4. Hatch rates (%) and times to 50% hatch for eggs of *R. tapirina* incubated at different salinities and temperatures ($\bar{x} \pm$ s.e., n=3 replicates).**

| Salinity | Temperature (°C) | | | | |
|---|---|---|---|---|---|
| (‰) | 6* | 9 | 12 | 15 | 18* |
| 15 | 0 | 50 (4)[a] | 38 (2)[a] | 33 (3)[b] | 0 |
| 25 | 0 | 55 (11)[a] | 50 (7)[a] | 41 (4)[b] | 0 |
| 35 | 0 | 47 (9)[a] | 59 (7)[a] | 31 (1)[b] | 6 (4) |
| 45 | 0 | 40 (1)[a] | 50 (2)[a] | 46 (2)[b] | 1 (1) |
| Mean time to 50% hatch (°h)** | N/A | 1,197 | 1,212 | 1,093 | 1,089 |

* Excluded from statistical analysis

** There was no variation in hatching times between replicates at the same temperature.

Figures sharing the same superscript are not significantly different (P>0.05).

**Table 3.3.5. Results of two-way ANOVA comparing the hatch rates (arc sin $\sqrt{}$ transformed data) of *R. tapirina* eggs incubated at different salinities and temperatures.**

| Source | DF | SS | F Ratio | P value |
|---|---|---|---|---|
| Temperature | 2 | 0.093 | 5.933 | **P < 0.05** |
| Salinity | 3 | 0.035 | 1.479 | **P > 0.05** |
| Temperature x Salinity | 6 | 0.108 | 2.285 | **P < 0.07** |

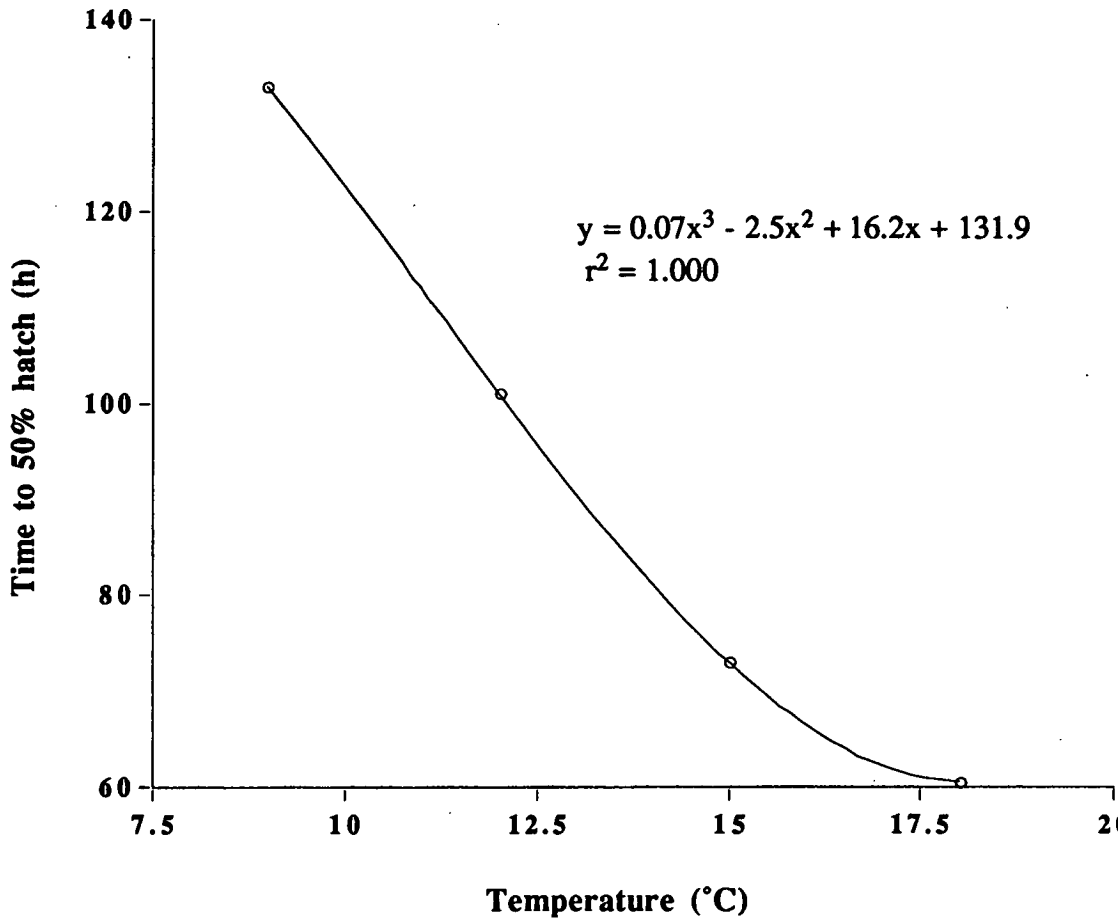


Fig. 3.3.1. Mean times to 50% hatch ($\bar{x} \pm$ s.e., n=12 replicates at each temperature, except 18°C at which only 3 replicates hatched) at different temperatures for eggs of *Rhombosolea tapirina* incubated at 35‰ (there was no variation in hatching times between replicates at the same temperature).

**(ii) Yolk-sac larvae**

*Experiment 1. Length and survival at completion of yolk absorption*

Temperature had a significant effect (P<0.05) on the length of *R. tapirina* larvae at the end of the yolk-sac stage, but had no effect on survival (Tables 3.3.7. and 3.3.8.). The final lengths of larvae decreased with increasing temperature above 15°C, but growth rates increased, reaching a peak at 15-18°C, before decreasing at 21°C. Larvae held at 18°C were significantly smaller (P<0.05) at the completion of yolk absorption, than those held at either 15, 12 or 9°C. There were no significant differences (P>0.05) in length between larvae held at 16.5, 15, or 12°C. There were no significant differences (P>0.05) in length between larvae held at 16.5 and 18°C. Survival was not significantly different (P>0.05) between treatments. The growth rates at 18 and 16.5°C were significantly (P<0.05) faster than at 12 or 9°C and the growth rates at 18 and 15°C were not significantly different. The growth rate at 12°C was significantly faster than that at 9°C. A high rate of deformities including one fish with a deformed jaw, was observed in the larvae held at 9°C but this was not significantly higher (P>0.05) than the rates observed in other treatments.

**Table 3.3.7. Length, survival, growth rate and rates of deformity of *R. tapirina* larvae held at different temperatures until the completion of yolk absorption ($\bar{x}$ ± s.e., n = 3 replicates, salinity=35‰).**

| Temperature (°C) | Mean length (mm) | Survival (%) | Growth rate (mm/d) | Time to yolk absorption (h)* | Rate of deformities (%) |
|---|---|---|---|---|---|
| 9 | 2.80 (0.04)$^{a}$ | 75 (8) | 0.08 (0.01)$^{a}$ | 144.6 | 18 (0.03) |
| 12 | 2.77 (0.02)$^{ab}$ | 73 (8) | 0.11 (0.01)$^{b}$ | 97.1 | 7 (0.04) |
| 15 | 2.76 (0.02)$^{ab}$ | 72 (8) | 0.11 (0.00)$^{bc}$ | 96.6 | 6 (0.03) |
| 16.5 | 2.73 (0.02)$^{bc}$ | 73 (8) | 0.13 (0.01)$^{d}$ | 76.3 | 2 (0.02) |
| 18 | 2.69 (0.02)$^{c}$ | 72 (8) | 0.12 (0.03)$^{cd}$ | 73.8 | 7 (0.04) |

Figures sharing a common superscript are not significantly different (P>0.05).

* There was no variation in times to yolk absorption, due to the method used (all fish were sampled at the same time).

**Table 3.3.8. Results of one-way ANOVA comparing the final lengths (arc sine √ transformed due to +ve skewed distribution), survival (arc sine √ transformed data), growth rates and rate of deformities (arc sine √ transformed data) of *R. tapirina* cultured through the yolk-sac stage at different temperatures (1 outlier removed from 9°C treatment. P<0.05, Grubbs test).**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 4 | 0.0000202 | 0.000005 | 3.8326 | **P < 0.05** |
| Error | 10 | 0.0000132 | 0.000001 | | |
| Total | 14 | 0.0000333 | | | |
| Survival | | | | | |
| Model | 4 | 0.0031 | 0.0008 | 0.0314 | **P > 0.05** |
| Error | 10 | 0.2454 | 0.0245 | | |
| Total | 14 | 0.2485 | | | |
| Growth | | | | | |
| Model | 4 | 7.51 | 1.88 | 17.04 | **P < 0.05** |
| Error | 10 | 1.10 | 0.11 | | |
| Total | 14 | 8.61 | | | |
| Deformities | | | | | |
| Model | 4 | 0.198 | 0.050 | 1.836 | **P > 0.05** |
| Error | 10 | 0.270 | 0.027 | | |
| Total | 14 | 0.468 | | | |

*Experiment 2. Growth rate and yolk absorption*

Growth rates decrease towards the end of the yolk-sac stage, with little variation between temperatures (Tables 3.3.9., 3.3.10. and Fig. 3.3.2.). The larvae at 21°C are the exception, showing very poor growth after 40-50 hours post-hatch and complete mortality after yolk absorption. There were no significant differences in length between larvae cultured at either 9, 12 or 15°C. Larvae cultured at 18°C were significantly smaller than those cultured at either 9, 12 or 15°C, but significantly larger than those cultured at 21°C. The fastest growth rates were recorded at temperatures of 18 and 15°C.

**Table 3.3.9. Length, time to oil droplet absorption and growth rate ($\bar{x} \pm$ s.d., n = 10 larvae) of *R. tapirina* larvae held at different temperatures until the completion of yolk absorption.**

| Temperature (°C) | Mean length at complete yolk absorption (mm) | Time to oil droplet absorption (h) | Growth rate (mm/d) |
|---|---|---|---|
| 9 | 3.20 (0.14)[a] | 184.5-208.5 | 0.14 |
| 12 | 3.12 (0.11)[a] | 143.5-163 | 0.17 |
| 15 | 3.18 (0.04)[a] | 120.5-143.5 | 0.22 |
| 18 | 3.00 (0.10)[b] | 101.5-120.5 | 0.22 |
| 21 | 2.75 (0.13)[c] | 101.5-120.5 | 0.15 |

At a temperature of 15°C, the yolk sac was fully absorbed at approximately 80 hours post-hatch and the oil droplet was fully absorbed at 120.5 hours post-hatch. First-feeding (data from section 3.3.3.) occurred after complete yolk absorption, but before the oil droplet was fully absorbed (Figs. 3.3.3. and 3.3.4.). The rate of yolk absorption accelerated with increasing temperatures above 15°C, but the time of first-feed was not established at these temperatures. The rate of oil droplet absorption was similar at all temperatures.

**Table 3.3.10. Results of one-way ANOVA comparing the final lengths of *R. tapirina* cultured through the yolk-sac stage at different temperatures.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Model | 4 | 1.29 | 0.322 | 28.51 | **P < 0.05** |
| Error | 45 | 0.51 | 0.011 | | |
| Total | 49 | 1.80 | | | |

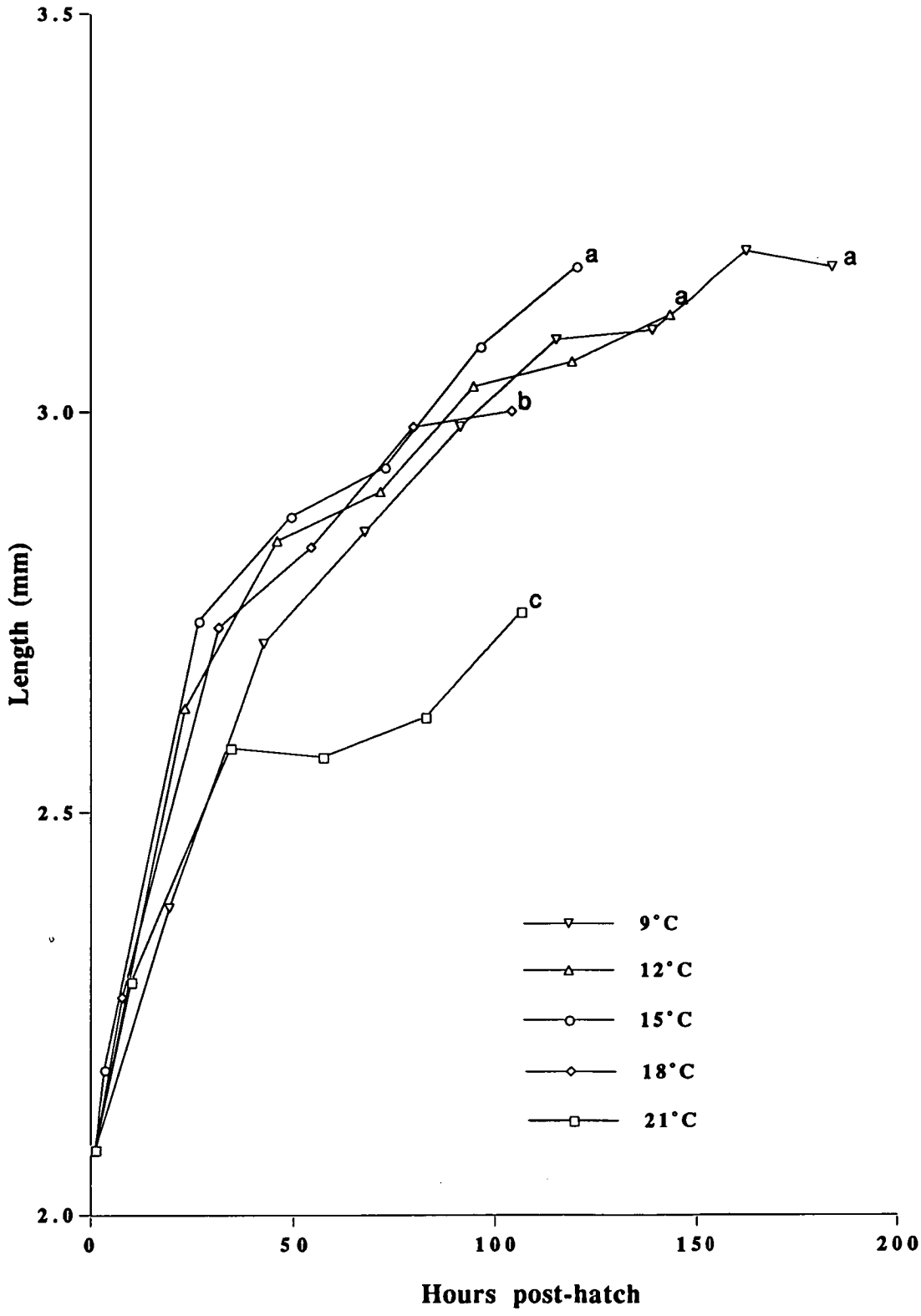


Fig. 3.3.2. Mean length (n=10 larvae) of *Rhombosolea tapirina* larvae from hatching to complete yolk absorption, at different temperatures (s.d., yolk absorption and oil droplet absorption, shown in Fig 3.3.3.).

Points sharing a common superscript are not significantly different (P>0.05).

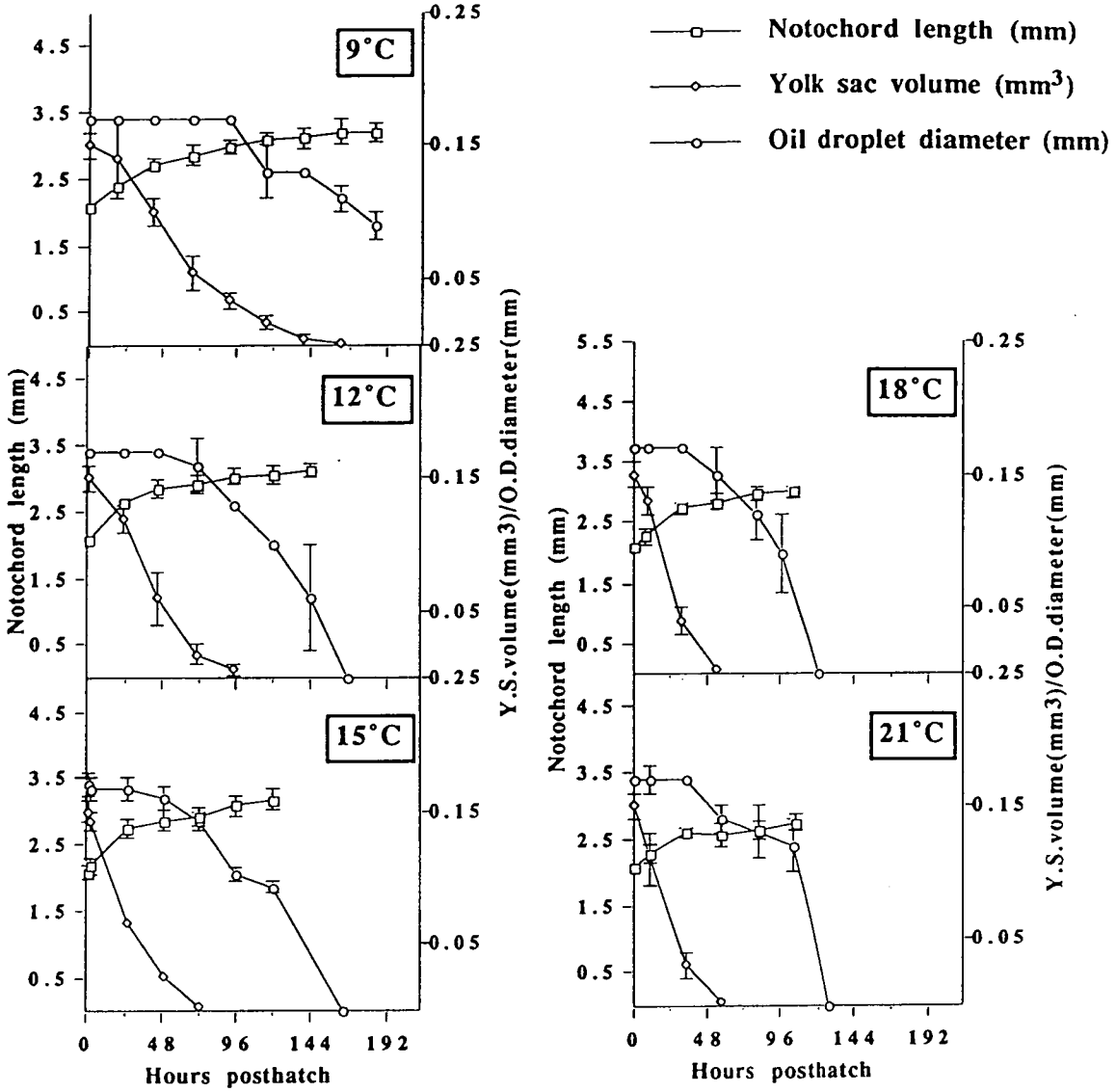


Fig 3.3.3. Growth in length and rate of yolk-sac and oil droplet absorption ($\bar{x} \pm$ s.d., n=10 larvae) for *Rhombosolea tapirina* larvae cultured at different temperatures. F.F. = time of first-feed from section 3.3.4.

Final samples taken on the last day oil droplet was present.

□ Experiment 1 ($\bar{x} \pm$ s.e., n=3 replicates).

o Experiment 2 ($\bar{x} \pm$ s.d., n=10 larvae).

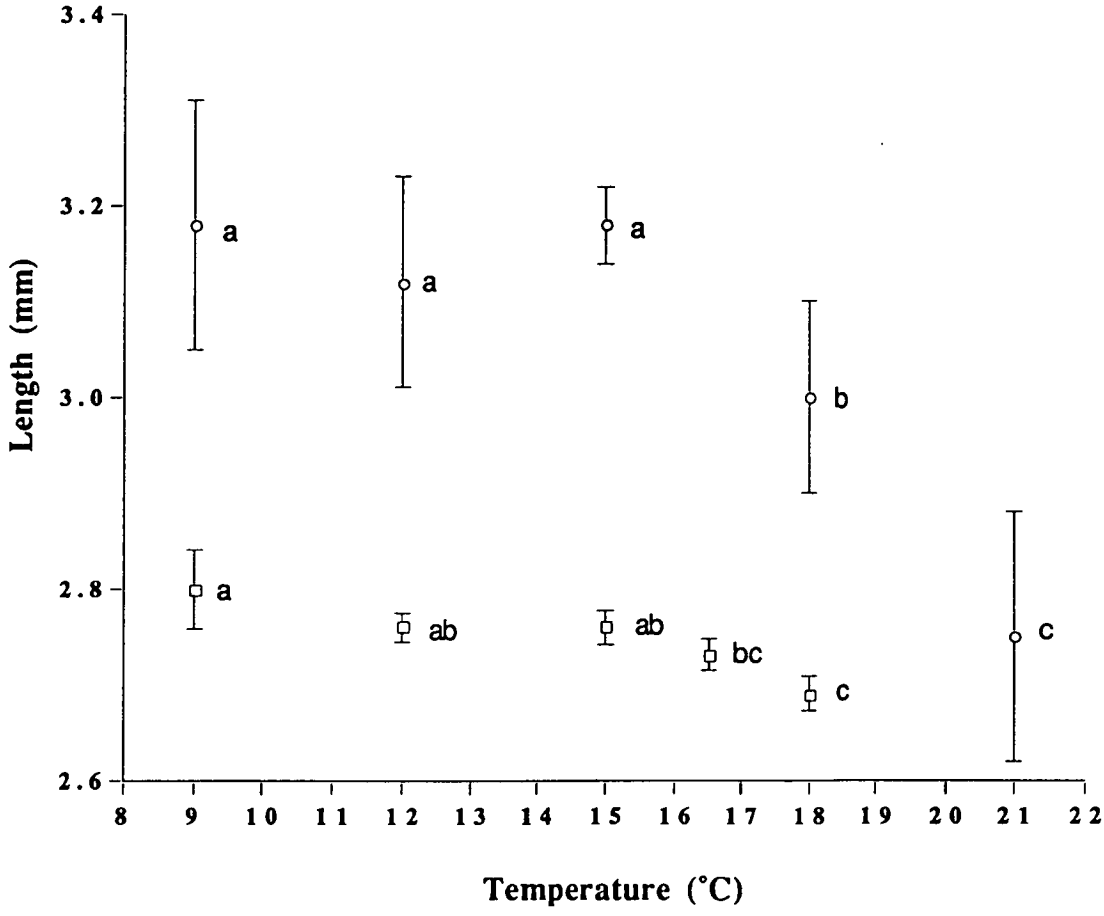


Fig. 3.3.4. Length ($\bar{x} \pm$ s.e. or s.d., see legend) of *Rhombosolea tapirina* larvae larvae incubated at different temperatures and measured at complete yolk absorption (Experiment 1) and complete oil droplet absorption (Experiment 2).

Points in each experiment sharing a common superscript are not significantly different (P>0.05).

### 3.3.4. Timing of first-feeding

The timing of first-feeding had a significant ($P<0.05$) effect on the growth of *R. tapirina* larvae and if delayed beyond day 4 post-hatch resulted in reduced growth. A delay beyond day 5 post-hatch, resulted in complete mortality (Tables 3.3.11., 3.3.12. and Fig. 3.3.5.). Mortalities of unfed larvae occurred from day 8 post-hatch onwards, as did those of larvae first-fed later than day 5 post-hatch. At day 13 post-hatch there were no survivors among fish first-fed on either day 6 post-hatch or later. The difference in length between larvae first-fed on days 3 and 4 post-hatch was not significant ($P>0.05$). However, larvae first-fed on day 5 post-hatch were significantly smaller ($P<0.01$) than those first-fed earlier.

**Table 3.3.11. Length and survival after 13 days, for *R. tapirina* larvae first-fed on days 3, 4 and 5 from hatching ($\bar{x} \pm$ s.e., n=3 replicates).**

| Day 1st Fed | Final mean length (mm) | Survival (%) |
|---|---|---|
| 3 | 4.71 (0.04)$^a$ | 65 (8) |
| 4 | 4.65 (0.04)$^a$ | 68 (10) |
| 5 | 4.15 (0.04)$^b$ | 48 (11) |

Figures in the same column sharing a common superscript are not significantly different ($P>0.05$).

**Table 3.3.12. Results of one-way ANOVA comparing the final lengths and survival (arc sine $\sqrt{}$ transformed data) of *R. tapirina* larvae first-fed on different days post-hatch.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 59.6 | 29.8 | 28.9 | **$P < 0.05$** |
| Error | 6 | 6.2 | 1.0 | | |
| Total | 8 | 65.8 | | | |
| Survival | | | | | |
| Model | 2 | 0.08 | 0.04 | 1.3 | **$P > 0.05$** |
| Error | 6 | 0.19 | 0.03 | | |
| Total | 8 | 0.27 | | | |

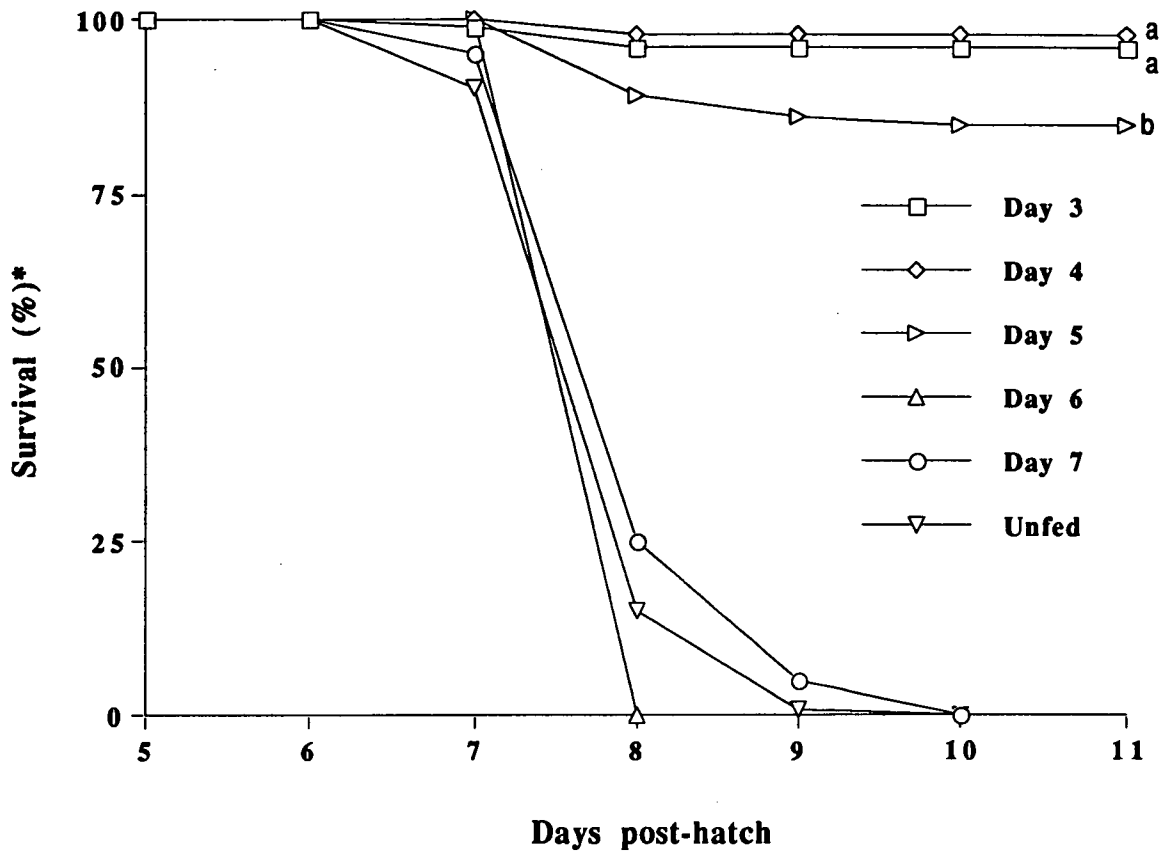


Fig. 3.3.5. Survival rates ($\bar{x} \pm$ s.e., n=3 replicates) of *Rhombosolea tapirina* larvae offered food on different days post-hatch (legend gives days post-hatch on which food was first offered).

Points sharing a common superscript are not significantly different (P>0.05), data for days 6, 7 and unfed were not analysed.

* survival on the graph is observed survival and is therefore different from actual survival due to the difficulty of counting mortalities. Actual survival is given in Table 3.3.11. and is the number of survivors divided by the initial number of larvae (50) x 100 i.e. $\frac{\text{survivors}}{50} \times 100$

Points with no visible error bars have very small s.e.

## 3.4. DISCUSSION

*Hatch rates under general incubation conditions*

The hatch rates of greenback flounder (*Rhombosolea tapirina*) eggs in the present study were low with an average of approximately 30-50%. With the Japanese flounder (*Paralichthys olivaceous*) a hatch-rate of 76% was achieved with water flow of 10-20 l/hour and with common flounder (*Limanda yokahamae*), hatch rates of 21-97% were obtained in a static seawater system (Kuronuma and Fukusho, 1984). Shelbourne (1975) records hatch rates of 80-95% with the eggs of plaice (*Pleuronectes platessa*) incubated in 54 l aquaria receiving a flow of 4 l/ hour filtered seawater. Person-Le Ruyet (1990) states that a 50% mortality of *S. maximus* eggs should be expected during the incubation phase in commercial hatcheries, due to various problems.

Fungal or bacterial infection of eggs was observed on only one occasion in the present study and it was therefore, not considered necessary to add antibiotics to the incubation water. This is possibly due to the short incubation period of *R. tapirina* eggs compared to other species of flatfish. Shelbourne (1975) observed that the eggs of *P. platessa* often became opaque and adhered to one another due to fungal infection, causing increased mortalities just prior to, and at hatching. By adding 50 I.U. sodium penicillin G and 0.05 g/ml streptomycin sulphate to the tanks, a higher survival was obtained (Shelbourne, 1975). Howell (1979) used 10 ppm tyrosine tartrate to reduce fungal and bacterial contamination, in a static system for turbot (*Scophthalmus maximus*) eggs.

Complete mortality of some batches of apparently good quality eggs, was observed on two occasions during the present study. This appeared to be due to poor water quality and bacterial contamination, coupled with the generally poor quality of the eggs. Better survival rates were obtained when water filtered to 1 µm was used for incubation. Static systems seemed to be adequate for small batches with low stocking densities (<200/l) but a very low water flow of approximately 45 l/hour was required for higher stocking densities. In the present study the eggs of *R. tapirina* were successfully incubated in artificial seawater, a method previously described by Corneille *et al.* (1989) and Baynes (1991) and found to be a highly successful method of eliminating bacteria and possible variations between batches of seawater.

Shelbourne (1975) experimented with the effects of stocking density on the eggs of *P. platessa* and found no differences between hatch rates at densities of up to 55.5 eggs/l. Subsequent larval survival actually increased with density up to metamorphosis, after which density dependant mortalities became apparent, suggesting an optimum

stocking density of 36-54 eggs/l. Devauchelle *et al.* (1986), calculated the optimum stocking density for the eggs of the sea bream (*Sparus auratus*), in 1 l incubators with a flow rate of 120 l/hour. No significant difference could be detected for densities of 1,000-10,000 eggs/l. However, the emergent larvae could not survive for more than 24 hours at the highest density. The maximum stocking density for the eggs of *S. maximus* and *S. solea* was considered to be 6/7 g eggs/l (approximately 6-7,000/l) (Devauchelle *et al.*, 1986). It appears that the main criteria for calculating the stocking density of eggs, is the optimum stocking density for the larvae when they hatch, as density has a negligible effect on egg development and hatch rates. It may be advantageous to use high stocking densities for egg incubation in a dedicated incubation system, which would require little water and space. The eggs could be transferred to a separate larval rearing system prior to hatching. This method is used for the commercial culture of *D. labrax* (Barnabé, 1990).

*Tolerance of eggs to physical disturbance*

In the present study the eggs of *R. tapirina* were tolerant of being removed from the water and could therefore be transferred from the incubation tanks to the rearing tanks and weighed to assess the number, at the same time. Holmefjord and Bolla (1988) found that the eggs of Atlantic halibut (*Hippoglossus hippoglossus*) could survive severe mechanical stress after the blastopore had closed (6 days post-fertilisation at 8°C). Maximum strength was observed in the chorion of cod (*Gadus morhua*) eggs after 24 hours, when the blastodisc was formed. Hatch rates were not measured in this study (Kjørsvik and Lønning, 1983). Pommeranz (1974) showed that mechanical resistance does not necessarily correlate with hatch rates and in *P. platessa* the mechanical resistance is high from about 10 hours post-fertilisation but viability is low until the embryo has surrounded about half of the yolk's circumference. After this stage the eggs become tolerant to mechanical stress.

*The effect of salinity and temperature on eggs*

In the present study it was found that the eggs of *R. tapirina* can tolerate salinities of between 15 and 45‰, after fertilisation, with no effect on subsequent hatch rates. However, salinity may have an effect on the eggs even before their release from the female. Fish kept in low salinity water prior to spawning, transfer water from the blood to the eggs via the ovarian fluid (Alderdice, 1988; Holliday, 1988). The eggs of *S. maximus* living in the Baltic Sea, show an adaptation to the low salinities found in this area and their eggs have a similar range of optimum salinity as their environment (Kuhlmann and Quantz, 1980). Once released the gametes themselves are fairly tolerant of salinity shocks (Holliday, 1988). In the present study the broodstock were

maintained in seawater of 35‰ salinity and this may have determined the optimum salinity range required for the eggs. However, there were no differences in hatch rates of eggs incubated in a wide range of salinities, only fertilisation rates were significantly affected and the optimum salinity was approximately that of the broodstock tank (section 2.3.3. (iii)). The water permeability of fish eggs prior to fertilisation is an order of magnitude higher than after fertilisation, due to some property of the membrane which is temporarily necessary for fertilisation (Riis-Vestergaard, 1987). Osmoregulation at the time of fertilisation is dependant on the permeability of the plasma membrane and is therefore effected by the osmolarity of the fluids in the egg (Alderdice, 1988). The osmolarity of the egg as decided by the salinity of the broodstock tank may therefore be optimal for fertilisation. This should be taken into account if broodstock are to be maintained at salinities other than those in which the eggs will be fertilised.

The optimal temperature for incubation of eggs was 9-12°C. Newell (1961) and Edwards (1979) both reported seawater temperatures of 12-13°C off the Tasmanian east coast during August and September. This is the natural spawning season of *R. tapirina* and it is therefore not surprising that the eggs are adapted to these temperatures.

The tolerance ranges for the eggs of *R. tapirina* were found to be salinities of 15‰ to 45‰ and temperatures of 9-18°C, a similar range to other flatfish species. The eggs of *S. maximus* from the Baltic Sea exhibit total mortality at salinities of less than 5‰ and temperatures below 10°C. Total mortality of sole (*Solea solea*) eggs occurred at temperatures of 22°C and the larvae failed to reach the surface for swim-bladder inflation at salinities lower than 10‰. The optimal temperature range was 10-16°C and the optimum salinity range was 20-40‰ (Fonds, 1979). Irvin (1974) set the tolerance range for *S. solea* eggs at 12-16°C. Holliday and Jones, (1967) recorded a high mortality for *P. platessa* eggs incubated at salinities of less than 17.5‰, with mortality generally occurring before the closing of the blastopore. High mortality (70-85‰) also occurred if eggs were transferred from normal seawater to water of low salinity before the blastopore was closed. However, it was not stated whether this was still a problem if eggs were transferred before first cleavage. Transfer of *R. tapirina* eggs to low salinities prior to first cleavage did not appear to affect the hatch rates in the present study.

The eggs of *R. tapirina* must be capable of osmoregulation after fertilisation. This is also the case with the eggs of *P. platessa* (Holliday and Jones, 1967). Holliday and Jones (1965) showed that osmotic regulation in the demersal eggs of the herring (*Clupea harengus*) is not achieved until after the closing of the blastopore. It is

suggested that pelagic eggs osmoregulate at an earlier stage than demersal eggs but the reason for this is unclear. Alderdice (1988) suggests that, directly after fertilisation, osmotic concentration is maintained by the impermeability of the plasma membrane and this role is taken over by the cells of the blastoderm as they overgrow the yolk. It is also suggested that chloride cells may be formed at the time of yolk plug closure in embryos with blastula cells that are intolerant of ion concentration extremes; *P. platessa*, and probably *R. tapirina*, appear to fall into this category. The eggs of S. *maximus* from the Baltic sea also showed greatest mortality during the late gastrula stage, in water of low salinity (Kuhlmann and Quantz, 1980).

In the present study, the eggs of *R. tapirina* showed a tendency towards higher survival at combinations of high temperature/high salinity and low temperature/low salinity. This is also the case for *Parophrys vetulis* (English sole), *C. harengus* and *S. solea* (Alderdice and Forrester, 1968; Alderdice and Velson, 1971; Devauchelle *et al.*, 1987). Alderdice and Forrester (1968) concluded that this was associated with north-south salinity-temperature trends found in the natural environment. However, Kuhlmann and Quantz (1980) found that, with eggs of *S. maximus* from the Baltic Sea, optimal hatch rates occurred with combinations of low salinity and high temperature or high salinity and low temperature.

The rate of development of *R. tapirina* eggs increased rapidly with temperature in a curvilinear fashion across the range of temperatures investigated in this study. Jones (1972) found the relationship between temperature and time to hatch, was linear with the eggs of *S. maximus* and *S. rhombus* (brill). However, Fonds (1979) observed development of *S. solea* eggs increasing rapidly up to 19°C in a curvilinear fashion as was shown with *R. tapirina.* A similar increase in development rate with temperature was observed by Jones (1972) with *S. maximus*. In the present study the time taken for 50% of *R. tapirina* eggs to hatch was 1,089-1,212°h which is similar to the time of 1,082-1,247°h, obtained by Crawford, (1984a). In many species, the time to hatch, as measured by °h, appears to decrease with increasing water temperature (Table 3.4.1.).

*The effect of temperature on yolk-sac larvae*

The optimal temperature for fastest growth during yolk-sac absorption in *R. tapirina* is 15°C. Culture at this temperature resulted in larger larvae (2.76-3.18 mm) at complete yolk and oil absorption (between 120.5 hours and 143.5 hours or 5.2-5.98 days) than at higher temperatures and also gave faster growth rates than at lower temperatures. The larvae of *S. maximus* exhibit the same development as *R. tapirina* and growth per unit of yolk absorbed is highest at 15.1°C; a length of 3.7-3.9 mm is reached on the yolk reserves (Jones, 1972). In most species this stage takes slightly longer, but it is

highly dependant on temperature. Ehrlich and Muszynski (1981), showed that larvae of the grunion (*Leuresthes tenuis*), actively seek water temperatures that minimise the duration of the yolk-sac stage and actively feeding larvae select temperatures that allow for maximum growth efficiency.

**Table 3.4.1. Times to 50% hatch for the eggs of a number of flatfish species**

| Species | Time to hatch (°h) | Temperature (°C) | Reference |
|---|---|---|---|
| Greenback flounder | 1,197 | 12 | This study |
| *R. tapirina* | 1,089 | 18 | |
| Greenback flounder | 1,247 | 12.5-12.7 | Crawford, 1984b |
| *R. tapirina* | 1,082 | 16.2-17.1 | |
| Japanese flounder | 882 | 16-20 | Kuronuma and |
| *P. olivaceus* | | | Fukusho, 1984. |
| Japanese plaice | 2,090 | 6.7 | Kuronuma and |
| *L. yokahamae* | 1,800 | 15 | Fukusho, 1984. |
| Atlantic halibut | 2,256 | 4.7 | Blaxter *et al.*, 1983 |
| *Hippoglossus hippoglossus* | 2,184 | 7 | |
| Sole | 1,550 | 10 | Fonds, 1979 |
| *S. solea* | 1,329 | 16 | |

During the yolk-sac stage the larvae of *R. tapirina* float passively at the water surface, with the yolk-sac uppermost but by the end of this stage, they are swimming more vigorously, the eyes are black, the body is more heavily pigmented and the digestive system is functional. Stages of development in *R. tapirina* are described fully by Crawford (1986). The change from endogenous to exogenous nutrition in *R. tapirina* occurs after complete absorption of the yolk-sac (approximately day 4 post-hatch at 15°C) and before the complete absorption of the oil droplet (approximately day 5 post-hatch at 15°C). There was some evidence of feeding behaviour in larvae just before complete yolk-sac absorption on day 3 post-hatch, but no difference in growth rate was shown in larvae first-fed on day 3 rather than on day 4 post-hatch. Kohno *et al.* (1988) identified a number of distinct phases in the early development of rabbitfish (*Siganus guttatus*) larvae. These involved an initial phase during which rapid larval growth occurred using the yolk reserves. A second phase of slow growth and organogenesis based on yolk reserves. A third stage during which exogenous feeding first occurred while the yolk-sac and oil droplet where still present. And a fourth phase during which the oil droplet was absorbed and exogenous food was also used. In larval *R. tapirina* these phases are less obvious. A faster growth rate is observed at the commencement of the yolk absorption phase at 15°C, although at other temperatures

there appears to be a greater reduction in the growth rate as the eyes become pigmented.

The oil droplet is absorbed from around 70 hours onwards as the eyes, mouth and gut are developed. The oil droplet provides nourishment while the feeding ability of the larvae improves. Feeding on exogenous food commences after the complete absorption of the yolk at around 96 hours, while a small quantity of oil still remains but this only lasts for a further 24 hours. Both Laurence (1973) and Quantz (1985) measured a slight deficit in the energy budget just prior to feeding in *Tautoga onitis* (tautog) and *S. maximus* larvae respectively. Laurence (1973) concluded that in species that showed high fecundity, it was unnecessary for the parents to provide full yolk reserves as low survival rates were affordable. *R. tapirina* is such a species (Crawford, 1984b).

In the present study, the final length of *R. tapirina* was shown to be greater and the growth rate was faster, if the yolk-sac larvae were incubated at 15°C. The larger size of the larvae at first-feeding would probably increase their ability to withstand a period of starvation. Hjort (1926) proposed the theory that fluctuations in year class strengths of marine fish were due to poor recruitment in certain years when hatching or the start of exogenous feeding, coincide with periods of low food abundance. May (1974) reviewed the literature on this subject and decided that it was not as clear cut as Hjort (1926) suggested, however the basic theory was probably correct. In a study of three tropical marine species, Bagarinao (1986) showed that there was a direct relationship between the size of the eggs, larvae, yolk-sac and mouth, and the time of complete starvation. However, Houde (1974) did not find this relationship in a study of three subtropical species. It therefore appears that other factors must also be important in determining the time of complete starvation. Environmental factors such as temperature, may have some effect.

McGurk (1984) analysed the published data for twenty five species of pelagic marine fish larvae and found a significant positive correlation between temperature and the age of irreversible starvation. Pinus (1984) sampled wild populations of tiulka (*Clupeonella delicatula delicatula*) in the Sea of Azov and found a clear correlation between water temperature and recruitment. Maximum recruitment occurred at an optimum temperature of 15°C. It was concluded that this was due to greater egg survival, but could also have been due to greater larval feeding ability. This relationship was also recorded by Dickson *et al.* (1984) for *G. morhua*. These authors found that recruitment increased in times of lower water temperature.

*The timing of first-feeding*

With many pelagic marine fish larvae, first-feeding occurs when the yolk is entirely absorbed (Blaxter and Ehrlich, 1984; Yin and Blaxter, 1987). Yin and Blaxter (1987) showed that in four species of temperate marine fish, the peak of feeding intensity occurred at the time of complete yolk absorption or 1 day later, even in fish that had previously not been offered food. However, some species are able to begin feeding well in advance of yolk absorption, a strategy which is thought to confer a considerable survival advantage where zooplankton show a patchy distribution (Houde, 1974; Laurence, 1978; Kohno *et al.*, 1988).

At a temperature of 15-16°C, the larvae of *R. tapirina* commence feeding on the 4th day or 96-120 hours post-hatch and if the first encounter with food occurs later than this, the result is smaller larvae and high mortality at day 8 post-hatch. Fish fed for the first time after day 6 or 145.5 hours post-hatch, showed complete mortality on day 8-10 post-hatch. This is the 'point of no return' identified by Blaxter and Hempel (1966). Laurence (1978) calculated that the 'point of no return' occurs after yolk absorption, at approximately 25% of the time to complete yolk absorption, regardless of temperature or species and the present work shows that this theory applies to *R. tapirina*. The 'point of no return' also appears to correspond to the time at which the oil droplet is used up and no further endogenous food reserves remain. Starving fish were observed lying on the base of the tanks and only moved when touched with the tip of a pipette. This phenomena is due to osmotic imbalance caused by starvation (Blaxter, 1981). The results of the present study show that a survival rate of up to 60% can be achieved through the first-feeding period. This compares with the rate of 40-80% recorded by Person-Le Ruyet *et al.* (1981) for *S. maximus*.

Even at the optimum temperature there are only between 24 and 48 hours during which the larvae of *R. tapirina* must find food (Table 3.4.1). This is shorter than for milkfish (*Chanos chanos*) or seabass (*Lates calcarifer*) but similar to rabbitfish (*Siganus guttatus*) cultured at 28°C (Bagarinao, 1986) and similar to the three species studied by Houde (1974) at 22-32°C. This author found that at optimal temperatures, there was more yolk remaining at the time of eye pigmentation and concluded that this would infer a survival advantage. However, in a laboratory study, Laurence (1978) found that, although the growth rates of larval *G. morhua* and *Melanogrammus aeglifinus* (haddock) were higher at certain temperatures, this had no effect on their ability to withstand periods of starvation. Presumably though, in a wild situation where food is distributed patchily, rather than in the laboratory where food is readily available, the largest and strongest larvae would be better able to find it (Laurence, 1973).

**Table 3.4.2. Time in days post-hatch, to reach complete yolk absorption (Y), first-feed (F), 'point of no return' (R) and the time available in which to initiate feeding (A), in a number of marine fish species.**

| Species | Y | F | R | A | Temp (°C) | Reference |
|---|---|---|---|---|---|---|
| *R. tapirina* | 5.2-5.98 | 4-5 | 6-7 | 2-3 | 15 | This study |
| *S. maeoticus* | 9.4 | | 12.5 | | 15.5 | Spectorova *et al.*, 1974 |
| *S. maximus* | 5 | 2.7-3.75 | 7 | 4.3-3.25 | 17.5 | Jones, 1972 |
| *C. harengus* [1] | 8 | 6 | 12-13 | 6-7 | 7.5 | Yin and Blaxter, 1987 |
| *C. harengus* [2] | 5 | 3 | 8 | 5 | 9.2 | Yin and Blaxter, 1987 |
| *G. morhua* | 7 | 5 | 11 | 6 | 6.9 | Yin and Blaxter, 1987 |
| *P. flesus* | 7 | 6 | 10 | 4 | 9.5 | Yin and Blaxter, 1987 |
| *C. chanos* | 5 | 2.25 | 3.25 | 1 | 28 | Bagarinao, 1986 |
| *L. calcarifer* | 5 | 1.3 | 2.5 | 1.2 | 28 | Bagarinao, 1986 |
| *S. guttatus* | 3 | 1.5 | 2.5 | 1 | 28 | Bagarinao, 1986 |

[1]. Clyde stock. [2]. Baltic stock

The results of the present study show that:

1. The eggs of *R. tapirina* are tolerant of considerable disturbance at the tail-bud stage and can be transferred between tanks in the absence of water with no subsequent effect on the hatch rate.

2. The eggs of *R. tapirina* are tolerant of a wide range of salinities but should be incubated at 35‰ as this gives greater buoyancy and makes the eggs easier to handle.

3. A temperature of 9-12°C is optimal for the incubation of *R. tapirina* eggs and results in maximum hatch rates.

4. Yolk-sac absorption in *R. tapirina* larvae is optimal at 9-15°C as this results in larger and stronger larvae at first-feeding. A temperature of 15°C results in the fastest rowth rates.

5. First-feeding of *R. tapirina* larvae on exogenous food, occurs between the time of complete yolk-sac absorption and the absorption of the oil droplet, the 'point of no return' occurs at day 6 post-hatch at 15°C, while mortality from starvation occurs on day 8-10 post-hatch.

# CHAPTER 4

# CONDITIONS FOR LARVAL REARING

## 4.1. INTRODUCTION

In Europe, the larvae of turbot (*Scophthalmus maximus*), sole (*Solea solea*), sea bream (*Sparus auratus*) and sea bass (*Dicentrarchus labrax*) are generally cultured in conical or hemispherical tanks of 0.3-20 $m^3$ at stocking densities of 50-100 larvae/l (Person-Le Ruyet, 1990; Barnabé, 1990). Recirculation systems are often used in order to maintain control over environmental variables such as temperature and salinity. Black tanks with a white base are commonly used in order to increase the feeding efficiency of the larvae and overhead lighting of 10-2,000 lux is supplied, often for 24 hours/day. The survival rates of the larvae up to metamorphosis, vary from 0-40% (Person-Le Ruyet, 1990; Barnabé, 1990).

In Japan the larvae of Japanese flounder (*Paralichthys olivaceus*), yellowtail (*Seriola quinqueradiata*), striped jack (*Caranx delicatissimus*) and red sea bream (*Pagrus auratus*) are generally cultured in indoor or outdoor tanks of 1-200 $m^3$ at stocking densities of 10-50/l. Lower stocking densities are used in the larger tanks. Tanks are constructed of concrete or fibreglass and covered with shade cloth to reduce the light intensity to 500 lux. Photoperiod control is not generally used but water temperatures are adjusted to suit the species being cultured. A bloom of marine *Chlorella* sp. is generally encouraged in the tanks before the larvae are introduced (Kuronuma and Fukusho, 1984; Watanabe and Nomura, 1990).

In Norway, an extensive production method was developed for the culture of cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*). At first-feeding the larvae are introduced into an enclosure or lagoon of 2,000-60,000 $m^3$, from which all predators have been removed using rotenone. Natural prey organisms provide optimal nutritional quality and the larvae are allowed to develop naturally. However, problems of prey reduction to sub-optimal levels have been encountered. Generally, survival rates have been from 0-15% in extensive ponds (Cachelou *et al.*, 1989; Van Der Meeren, 1991). Although a much less costly method of producing marine fish than the normal intensive systems, the success of such extensive systems is highly dependant on site selection. Van Der Meeren (1991) also describes a method in which plastic bag enclosures of 50-200 $m^3$ are used for larval rearing. The larvae are fed with wild-harvested zooplankton which are filtered to the optimal size. Survival rates of 40-50% have been observed using this system for *H. hippoglossus*. Using this technique, one commercial operation was able to produce approximately 70,000 *S. maximus* juveniles in one season. However, the introduction of pathogens, with the wild zooplankton, can be a major problem with this culture method (B. Urup, pers. comm.).

In the commercial culture of turbot *S. maximus* and sole *S. solea* (Person-Le Ruyet, 1990), continuous light is currently used and has been shown to increase the growth rate of a number of fish species; it is usually coupled with increased survival (Tandler and Helps, 1985; Duray and Kohno, 1988; Villarreal *et al.*, 1985). Marine fish larvae are visual feeders and the longer the daylight period the more time there is available for feeding (Blaxter, 1968; 1969). However, there may be interactions with other variables such as light intensity and prey density which render long daylight periods ineffective in increasing the growth rate of some species (Barahona-Fernandes, 1979; Dowd and Houde, 1980).

Temperature regimes used for larval rearing are generally higher than those used for egg incubation and yolk absorption. Although, high rearing temperatures can result in reduced survival as well as increased growth (Johnson and Katavic, 1986). The optimal temperature for larval rearing of *S. maximus* and *S. solea* is 18-20°C (Person-Le Ruyet, 1990) and that for *P. olivaceus* 17-20°C (Watanabe and Nomura, 1990). Wild caught *R. tapirina* juveniles preferred temperatures of 11.3-15°C when placed in a temperature gradient (Crawford, 1984b) and the adults have a critical thermal maximum of 32.6°C (Roper, 1979). Jenkins (1987) studied the growth rate of wild caught *R. tapirina* larvae at 9-10°C, using the growth increments on the otoliths of larvae. The growth rate was higher in the wild (5.5 mm at day 30) than that obtained by Crawford (1984a) in the laboratory (less than 5.0 mm at day 30), even though temperature and food densities were lower in the wild at the time of sampling.

Although salinities of around 35‰ are generally used for marine fish larviculture, Crawford (1984b) found that wild-caught juveniles of *R. tapirina*, moved rapidly towards freshwater when placed in a salinity gradient. Larvae at stage 5 M (for a description of stages Appendix 1.5.2.) showed a bimodal distribution in a salinity gradient with 30% in the range 0-5‰ and 35% in the range 32-34‰. The larvae of plaice (*Pleuronectes platessa*) become less tolerant of upper and lower levels of salinity after metamorphosis, but can still survive salinities of 2.5-45‰ for up to a week (Holliday and Jones, 1967).

The specific objectives of the present study were:

1. To investigate intensive larval rearing systems for experimental work on larval marine fish, in order to evaluate their suitability for use in future studies.

2. To investigate the effect of different photoperiods on the larvae of *R. tapirina* from hatching to day 20 post-hatch, in order to maximise the larval feeding opportunity during first-feeding, and maximise growth and survival.

3. To investigate the effect of temperature on the growth rates of larval *R. tapirina* during the period up to and beyond metamorphosis, in order to establish the optimum temperature for maximum growth and survival.

4. To investigate the effect of different salinities on the growth and survival of metamorphosing *R. tapirina* larvae, in order to establish the optimal salinity for maximum growth and survival.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Systems

Incubation of eggs and mass-rearing of larvae was carried out in a system containing three 160 l, black, hemispherical, fibreglass tanks, serviced by a biological filter (Appendix 1.3). Light intensities of 500-600 lux were supplied for 24 hours/day; a temperature of 15°C and salinity of 35‰ were used; and larvae were stocked at approximately 50/l. Water flow was generally static for the first 10-15 days post-hatch and 45 l/hour thereafter. A measure of survival was obtained for some batches, by counting the remaining larvae after metamorphosis, but this was only an approximation due to the difficulty of counting the eggs accurately, at the time of initial stocking in the rearing tanks.

Two systems were constructed for small-scale experimentation on larvae (Hart *et al.*, 1994) (Fig. 4.2.2.). The holding tanks consisted of 3 l transparent plastic hemispherical goldfish bowls, painted on the outside with matt black paint, leaving an unpainted disc at the base to enable observation of larvae and mortalities. The seawater inlet consisted of a rigid black 4 mm I.D. domestic irrigation riser pipe, which was positioned in the centre of each tank and extended to the bottom. A 90° elbow at the top brought this inlet pipe through the side of the tank above the water level where it joined the main 13 mm PVC feed pipe. A 4 mm tap on the inlet pipe allowed the water flow rate of individual tanks to be adjusted. The outlet for each tank consisted of a 13 mm elbow inserted into the side of the tank through a rubber grommet at the desired water level. A piece of perforated 13 mm pipe covered with a fine screen material could be attached to this elbow on the inside of the tank to retain live food and larvae. An external 13 mm PVC pipe carried water to the common outlet pipe. A set of screens of any size mesh could be constructed and changed over, or cleaned easily. Short transparent sections were incorporated into both the inlet and outlet pipes so that water flow could be easily observed. Tanks were positioned in sets of three with each set having an individual biofilter/reservoir (Fig. 4.2.3.), this enabled

experiments involving salinity or temperature to be conducted without mixing occurring between systems. By connecting the standpipes of reservoirs and bypassing excess water to adjacent biofilters it was a very simple operation to join the whole system during experiments requiring a common water supply e.g. experiments involving photoperiod, light intensity.

The effluent pipes were divided before entering the biofilter and the water flow rate in each line was individually controlled by a 13 mm PVC tap. One of these lines flowed directly into the biofilter while the other flowed to the biofilter via a fine-mesh screen. When necessary the effluent water could be diverted through this fine-mesh screen to filter out uneaten live feeds before entering the biofilter. Normally the live feed circulated throughout the system ensuring an even distribution of prey organisms throughout the tanks. In cases where enrichment diets were being tested, or where it was undesirable for the feeds from different treatments to be mixed, the system could be split into individual sets (Chapter 5). Alternatively, a suitable pore size could be selected for the outlet screens on individual tanks, to ensure that the feeds were retained within the tanks (Fig. 4.2.1.).

The biofilter/reservoir was made up of a 20 l white bucket with a 10 l bucket sitting inside it suspended from the rim (Figs. 4.2.2. and 4.2.3.). The 10 l bucket contained a plastic artificial substrate as a biofiltration medium. Water from the tanks trickled through this substrate from a perforated ring of pipe fitted into the lid and flowing onto a plastic drip-plate. The reservoir (20 l bucket) was filled up to the base of the biofilter (10 l bucket), thus the total volume of each set of three tanks was approximately 19 l. A small aquarium heater could be placed in each reservoir to adjust the water temperature. A transparent 4 mm tube inserted into the base of the reservoir bucket and attached to the top with an open end, functioned as a water level gauge. A 13 mm tap at the base of the bucket enabled rapid draining of the system for water exchanges or cleaning. A small centrifugal aquarium pump (AquaClear 802 powerhead, 25 l/min) was positioned in each reservoir to supply water to the tanks. A bypass system with a tap allowed excess water to flow directly back to the reservoir/biofilter. This arrangement enabled the biofilters to be acclimatised prior to using the tanks and also made it possible to keep the biofilters running while the systems were not in use.

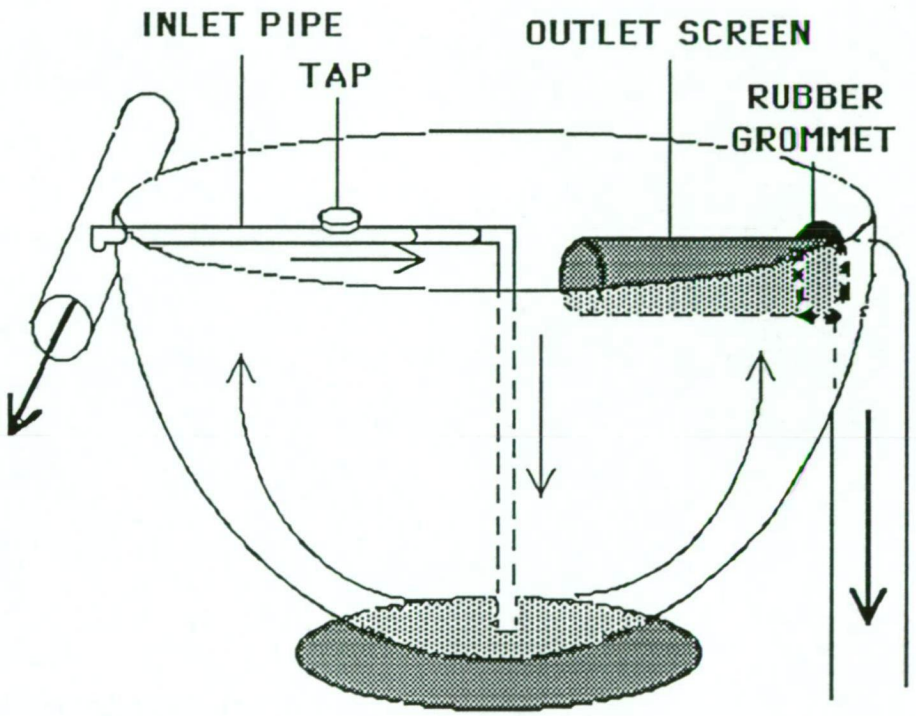


Fig. 4.2.1. Schematic diagram of one tank in the 3 l system used for experiments in Chapter 4. Arrows show direction of water flow (from Hart *et al.*, 1994).

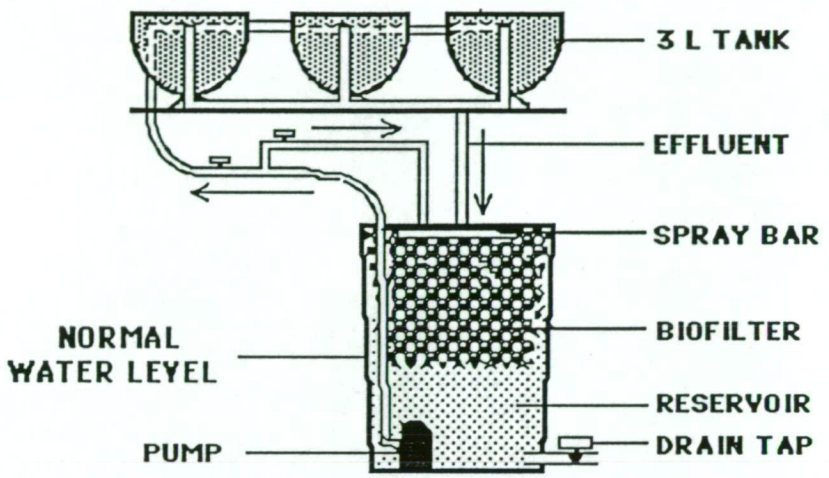


Fig. 4.2.2. Schematic diagram (end view) of the 3 l system used for larval rearing experiments in Chapter 4. Arrows show direction of water flow (from Hart *et al.*, 1994).

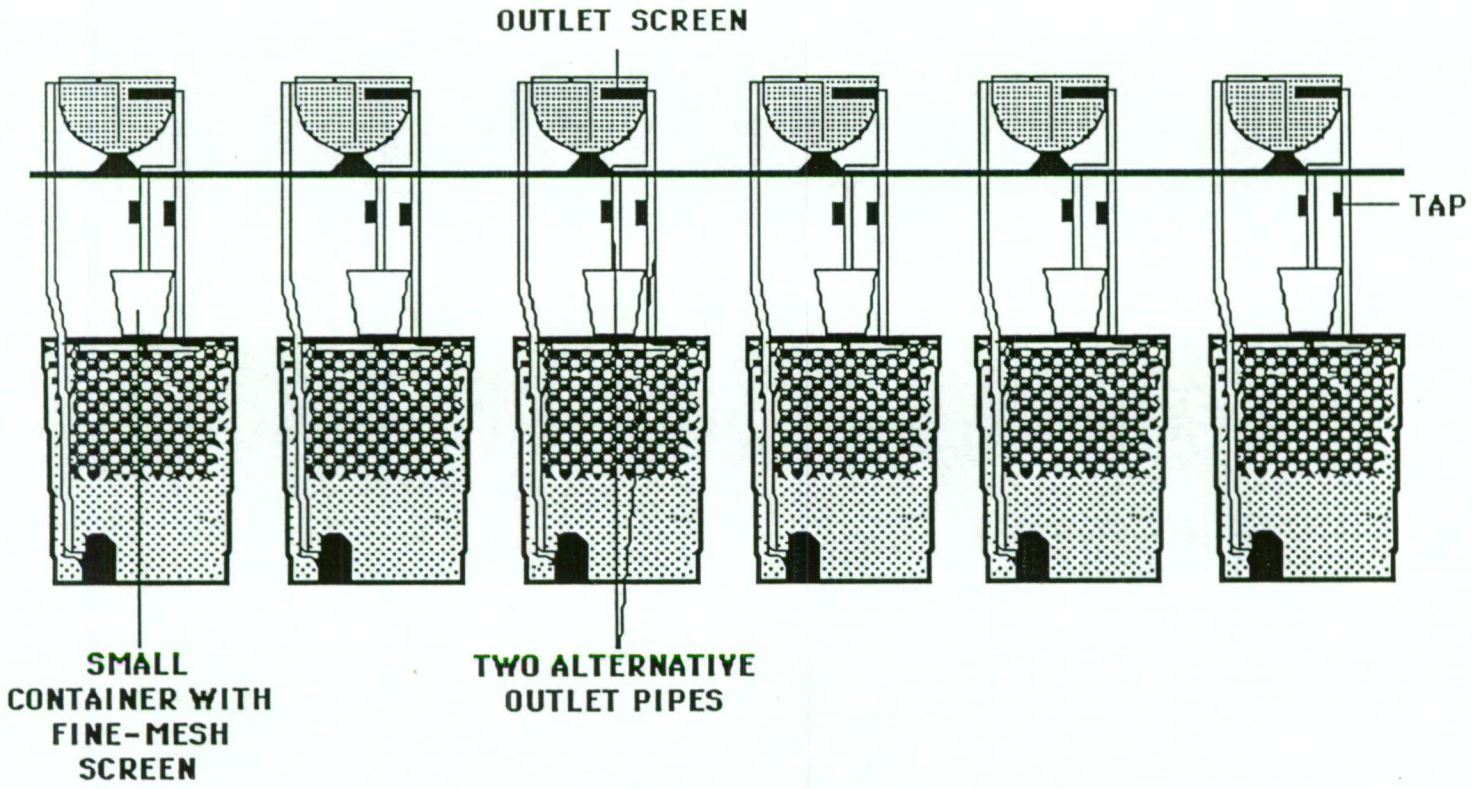


Fig 4.2.3. Side view of 3 L larval rearing system.

The whole system was constructed on a wooden bench of 1m x 1m x 1.6 m, in a temperature and photoperiod-controlled room. Temperature was maintained at 15°C and light intensity, measured at the water surface, was 300-400 lux.

### 4.2.2. The effect of photoperiod

In order to investigate the effect of photoperiod on the growth and survival of *R. tapirina* larvae, a set of eighteen, 3 l, black, hemispherical tanks in a recirculation system (Section 4.2.1.), were each stocked with one hundred and fifty newly-hatched larvae (initial length = 2.43 ± 0.04 mm; $\bar{x}$ ± s.e., n=50 larvae). The system was static until feeding commenced (day 4 post-hatch), after which the flow rates were maintained at 6 l/hour. Each set of three replicates was covered by a lightproof box containing a single Thorn EMI 18 Watt fluorescent tube, mounted 400 mm above the water surface, giving a mean light intensity of 1,699 ± 46 lux ($\bar{x}$ ± s.e.; n=15) this was higher than in other experiments, but appeared not to have a detrimental effect on the feeding ability of the larvae, (Section 4.3.2.). Photoperiod treatments were as follows:

1. Continuous light
2. 18 hours light : 6 hours dark per day.
3. 9 hours light : 3 hours dark + 9 hours light : 3 hours dark per day
4. 12 hours light : 12 hours dark per day
5. 6 hours light : 18 hours dark per day
6. Continuous dark

Larvae were fed twice daily with rotifers enriched with microalgae (Appendix 1.2). In order to maintain high nutritive levels, uneaten rotifers were flushed from the system for two hours prior to each morning feed. Feed density was maintained as closely as possible to 10 rotifers/ml, with the rotifers circulating freely throughout each system (preliminary trials showed that live prey organisms maintained a satisfactory level of survival when allowed to pass out of the tanks, through the biological filter, into the reservoir and pumped back into the tanks). Larval length was measured and survival calculated at days 10 and 20 post-hatch. The experiment was terminated at day 20 post-hatch.

### 4.2.3. The effect of temperature

To investigate the effect of temperature on the growth and survival of larval *R. tapirina*, a batch of fifty larvae of 11 days post-hatch (initial length = 4.10 ± 0.05 mm; $\bar{x}$ ± s.e., n=30 larvae), were randomly distributed in each of eighteen, 3 l, black,

hemispherical tanks (Section 4.2.1.). Each of the six reservoirs contained a Jäeger aquarium heater adjusted to the required temperature. The system was arranged so that each set of three tanks could be set at a different temperature. Temperature was measured twice daily in the tanks and the actual temperatures were calculated by taking the mean of all the temperature readings for each treatment. Temperatures were set at 15 (15.2± 0.1), 16 (16.6 ± 0.1), 17 (17.2 ± 0.1), 18 (18.8 ± 0.04), 19 (18.9 ± 0.02) and 20°C (19.9 ± 0.03) (recorded $\bar{x}$ ± s.e., n=58 readings). The inlet pipe of the 15°C tanks was passed through a water bath set at 12°C to reduce the temperature.

The larvae were fed once daily with *Artemia* enriched with Frippak Booster (Sanofi Aquaculture, Paris, France; Appendix 1.2.). The feeding rate was high enough to provide an excess of *Artemia* in the tanks. Water changes were carried out every second day by emptying the reservoir and refilling with 15°C seawater. The low flow rate (6 l/hour) into the tanks allowed this new water to heat to the desired temperature in the reservoir, before the tank water temperature was affected. Approximately every 7 days, a sample of ten larvae was removed, by pipette, from each tank, for length measurement. The pipette was made from silicon tube to avoid damaging the larvae. The experiment was terminated after 29 days and a sample of twenty larvae was removed from each tank for weight and length measurement. All remaining larvae in each tank were counted to enable calculation of the survival rate. Growth rate was calculated as the percentage length increase per day (%/day), using the formula:

$$\text{Growth rate (\%/day)} = \frac{\text{Final length - Initial length}}{\text{time in days}} \times 100 \text{ (Hopkins, 1992).}$$

### 4.2.4. The effect of salinity

To investigate the effect of salinity on the growth and survival of larval *R. tapirina*, fifty metamorphosing flounder of 34 days post-hatch (initial length = 6.5 ± 0.09 mm; $\bar{x}$ ± s.e., n=40 larvae) were randomly distributed in each of nine, 3 l, black, hemispherical, tanks (Section 4.2.1.) at a salinity of 35‰. Flow rates were maintained at 6 l/hour. Temperature was set at 15-16°C and continuous light was provided by overhead fluorescent tubes at an intensity of 300-400 lux. The salinity in two sets of three replicate tanks was reduced to 25‰ and 15‰ respectively, over the following 24 hours. Feeding was carried out twice daily, with microalgal-enriched *Artemia* (Appendix 1.2.), at a rate of 4-5 animals /ml/day introduced in two feeds. The experiment was terminated after 30 days and all remaining fish were measured.

## 4.3. RESULTS

### 4.3.1. Systems

A survival rate of approximately 15% was achieved from fertilisation to metamorphosis in the 160 l systems using continuous light, a temperature of 15°C and a salinity of 35‰. These larger systems were easier to manage than the 3 l system. However, management of the 3 l system was also relatively easy. Water exchanges were carried out as required, depending on the results of regular water sample analyses. While the systems were running, only a very small percentage (approx. 0.02%) of water flowed through the tanks while the majority of the 25 l/min available, was recirculated through the biofilter. This ensured maximum biofiltration capacity and ammonia was never a problem during experiments on larvae being fed live feeds (Appendix 1.1.). Flow rates were normally adjusted to around 0.05-0.15 l/min/tank and stocking densities ranged from 50-150 fish per 3 l tank (17-50 fish /l).

The system experienced only one failure which occurred during a weaning experiment when the solids and dissolved organic matter built up before the biofilter had fully acclimatised. Another cause of unnatural mortality occurred when fish became entangled in the outlet screen material. This problem was later overcome by using a static system, or intermittent water flow until the fish were sufficiently robust. Using tighter screens so that no folds were present to trap the larvae, also helped. Trials with striped trumpeter (*Latris lineata*) were unsuccessful in this system, due possibly, to the difficulty of preventing oil-film formation and subsequent malformation of the swim-bladder. However, this is a difficult species and even in normal-sized tanks, has only been successfully reared once (W. Hutchinson, unpublished data).

### 4.3.2. The effect of photoperiod

Increasing daylength resulted in improved growth rates of *R. tapirina* larvae, but had no effect on survival as long as at least some daylight was provided (Tables 4.3.1., 4.3.2. and Fig. 4.3.1.). Continuous light, or 18 hours of daylight broken into two periods of 9 hours, gave significantly better growth ($P<0.05$) than either 12 or 6 hours light, during the first 10 days post-hatch. However, the growth rates of larvae provided with 18 hours of light unbroken, were not significantly different ($P>0.05$) from those given either, 18 hours light broken into two periods of 9 hours, or 12 hours light.

Larvae cultured under total darkness or a 6 hour photoperiod showed significantly lower growth ($P<0.05$) than all other treatments at day 10 post-hatch. At day 20 post-hatch there were no significant differences in length ($P>0.05$) between larvae provided

with either, 24 hours light, 18 hours light, or broken 18 hour photoperiods. Fish maintained under a 12 hour photoperiod were significantly smaller ($P<0.05$) than those in all other treatments except 6 hours, which were significantly smaller ($P<0.05$) than those in all other treatments.

**Table 4.3.1. Length and survival rates of *R. tapirina* larvae cultured under different photoperiods ($\bar{x} \pm$ s.e., n=3 replicates).**

| Daylength (h) | Length at day 10 (mm) | Survival at day 10 (%) | Length at day 20 (mm) | Survival day 10 to day 20 (%) |
|---|---|---|---|---|
| 24 | 4.04 (0.05)$^{a}$ | 30 (12) | 6.50 (0.11)$^{a}$ | 73 (3) |
| 18 | 3.81 (0.01)$^{ab}$ | 13 (3) | 6.43 (0.13)$^{a}$ | 82 (5) |
| 9:3+9:3 | 3.89 (0.10)$^{ab}$ | 11 (3) | 6.53 (0.03)$^{a}$ | 46 (24) |
| 12 | 3.66 (0.03)$^{b}$ | 27 (9) | 6.01 (0.06)$^{b}$ | 67 (18) |
| 6 | 2.99 (0.07)$^{c}$ | 16 (4) | 4.30 (0.07)$^{c}$ | 51 (16) |
| 0 | 2.96 (0.00)$^{c}$ | 0.2 (0.2) | 0 | 0 |

Figures in the same column sharing a common superscript are not significantly different ($P>0.05$)

By day 10 post-hatch only one fish remained in the tanks without illumination and at day 20 this fish was also dead. However, there were no significant differences ($P>0.05$) in survival rates between other treatments. Survival rates were generally quite low during the first 10 days post-hatch, due to poor egg quality and reduced first-feeding ability (Chapter 3).

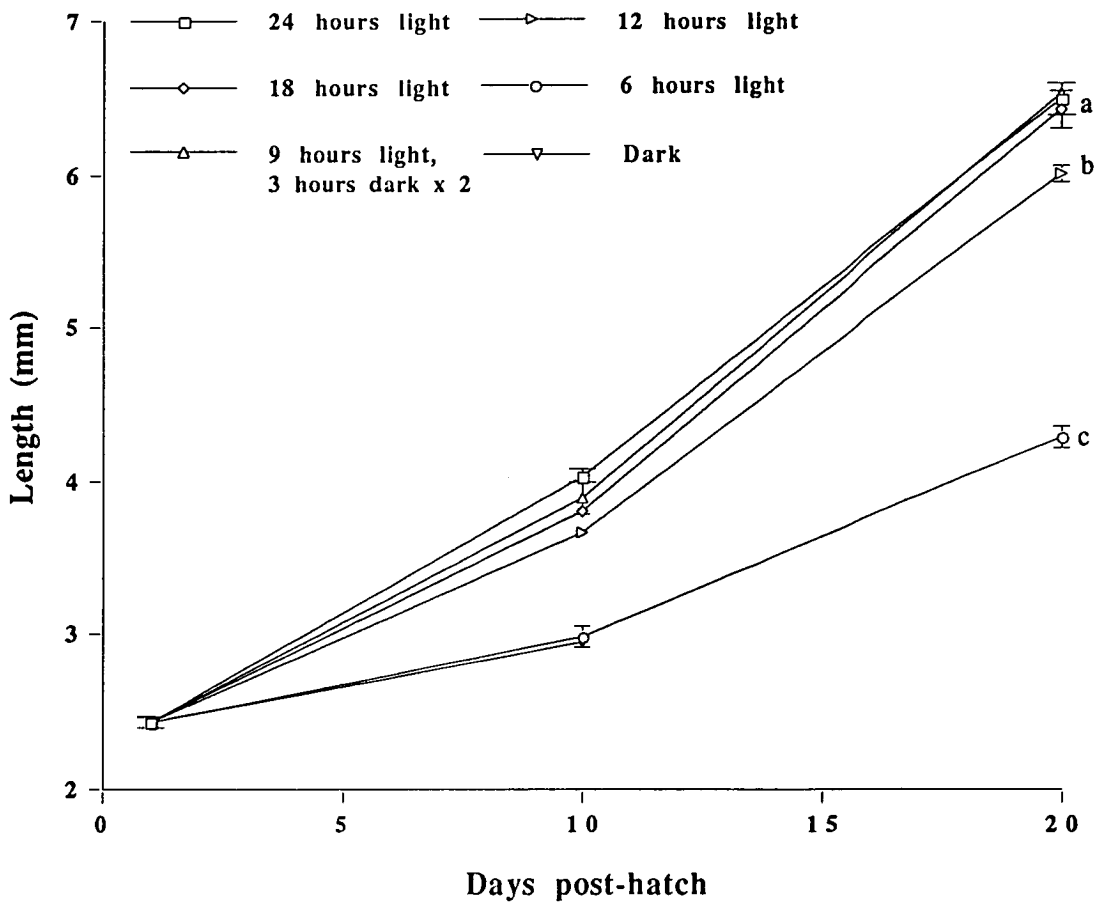


Fig 4.3.1. Growth in length ($\bar{x} \pm$ s.e., n=3 replicates) of *Rhombosolea tapirina* larvae cultured through first-feeding to day 20 post-hatch under different photoperiod regimes.

Points sharing a common superscript are not significantly different (P>0.05)

Points with no visible error bars have very small s.e.

**Table 4.3.2. Results of one-way ANOVA comparing the length at days 10 and 20 post-hatch and the survival rate (arc sine √ transformation) from day 10 to day 20 post-hatch, of *R. tapirina* larvae cultured under different photoperiod regimes**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Length at day 10 | | | | | |
| Model | 5 | 2.492 | 0.498 | 44.091 | **P < 0.05** |
| Error | 10 | 0.113 | 0.0113 | | |
| Total | 15 | 2.605 | | | |
| Length at day 20 | | | | | |
| Model | 4 | 10.391 | 2.598 | 105.376 | **P < 0.05** |
| Error | 9 | 0.222 | 0.025 | | |
| Total | 13 | 10.613 | | | |
| Survival from day 10-20 | | | | | |
| Model | 4* | 0.45 | 0.113 | 0.95 | **P > 0.05** |
| Error | 10 | 1.18 | 0.118 | | |
| Total | 14 | 1.63 | | | |

* Data for 24 h dark excluded due to variance = 0 causing heterogeneity of variance.

### 4.3.3. The effect of temperature

The growth rate of *R. tapirina* larvae increased significantly (P<0.05) with increasing temperature up to 18°C (Tables 4.3.3., 4.3.4. and Fig. 4.3.2.). However, survival over the 29 day period was unaffected. The longest larvae and fastest growth rates occurred at temperatures of 18-20°C. The final lengths and weights of larvae reared at 18, 19 and 20°C were not significantly different (P>0.05). Larvae cultured at 16 and 17°C were significantly shorter (P<0.05) in length than those cultured at 18, 19 and 20°C. Larvae cultured at 15°C were significantly shorter than all other treatments (P<0.05). Larvae cultured at 16°C were not significantly different (P>0.05) in weight from those cultured at 18°C. Larvae cultured at 17°C were not significantly different (P>0.05) in weight from those cultured at 15°C or 16°C. There were no significant differences in survival (P>0.05).

**Table 4.3.3. Final lengths, weights and survival data for *R. tapirina* larvae cultured at different temperatures ($\bar{x} \pm$ s.e., n=3 replicates).**

| Temperature (°C) | Final length (mm) | Final weight (g) | Growth rate (%/day) | Survival (%) |
|---|---|---|---|---|
| 15 | 11.24 (0.15)$^{a}$ | 0.022 (0.001)$^{a}$ | 25 | 85 (2) |
| 16 | 12.69 (0.18)$^{b}$ | 0.033 (0.001)$^{ab}$ | 30 | 73 (9) |
| 17 | 12.14 (0.17)$^{b}$ | 0.027 (0.001)$^{a}$ | 28 | 93 (2) |
| 18 | 13.70 (0.19)$^{c}$ | 0.037 (0.001)$^{bc}$ | 33 | 89 (7) |
| 19 | 14.06 (0.19)$^{c}$ | 0.040 (0.002)$^{c}$ | 34 | 86 (3) |
| 20 | 13.99 (0.21)$^{c}$ | 0.040 (0.002)c | 34 | 85 (2) |

Figures in the same column sharing a common superscript are not significantly different (P>0.05).

**Table 4.3.4. Results of a one-way ANOVA comparing the final lengths, weights and survival, of *R. tapirina* larvae cultured at different temperatures.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 5 | 19.482 | 3.897 | 18.303 | **P < 0.05** |
| Error | 12 | 2.555 | 0.213 | | |
| Total | 17 | 22.037 | | | |
| Final weight | | | | | |
| Model | 5 | 0.00075 | 0.00015 | 12.444 | **P < 0.05** |
| Error | 12 | 0.00014 | 0.000012 | | |
| Total | 17 | 0.00089 | | | |
| Survival | | | | | |
| Model | 5 | 0.121 | 0.024 | 1.785 | **P > 0.05** |
| Error | 12 | 0.163 | 0.014 | | |
| Total | 17 | 0.284 | | | |

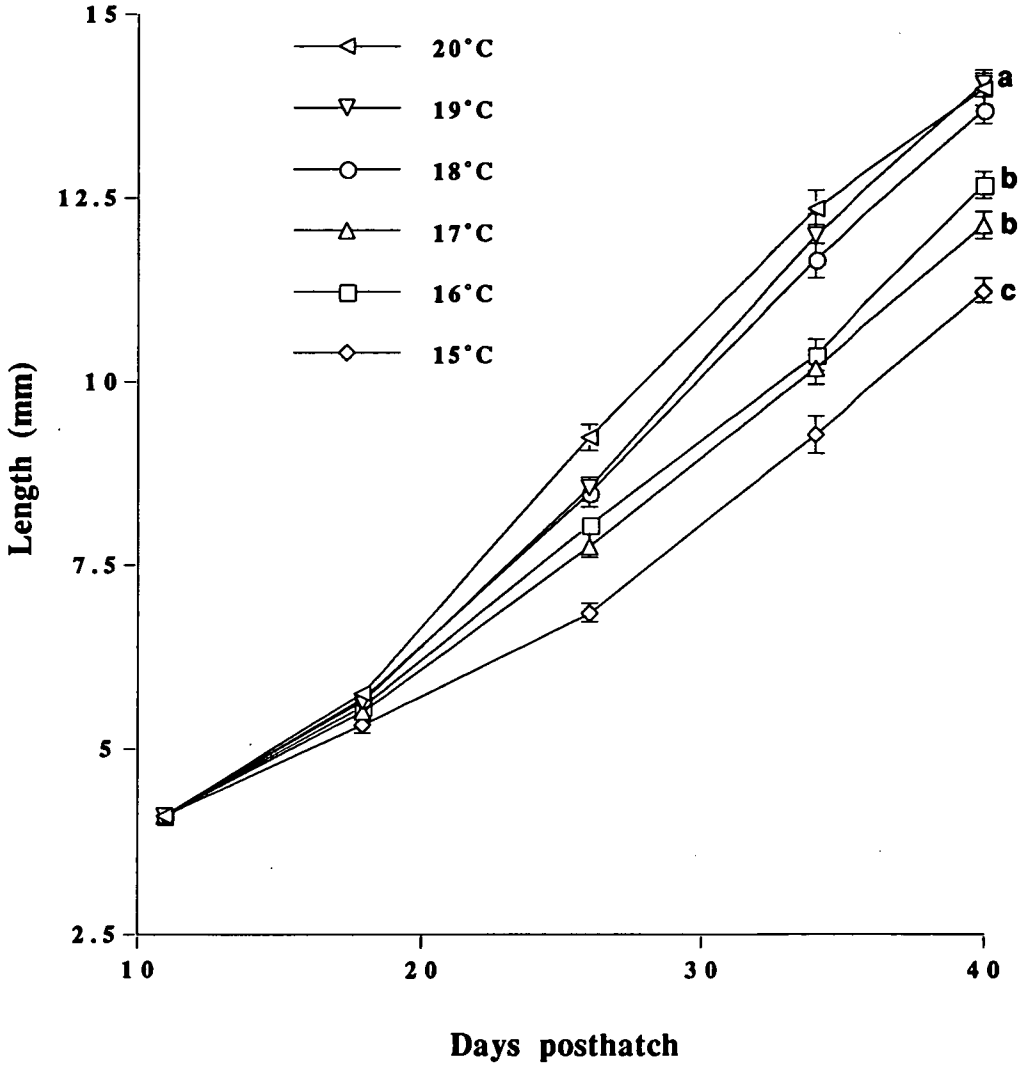


Fig. 4.3.2. Growth in length ($\bar{x}$ ± s.e., n=3 replicates) of *Rhombosolea tapirina* larvae cultured for 29 days at different temperatures.

Points sharing a common superscript are not significantly different (P>0.05).

Points with no visible error bars have very small s.e.

### 4.3.4. The effect of salinity

No significant differences in growth were observed when *R. tapirina* were cultured in seawater of different salinities for 30 days (Tables 4.3.5. and 4.3.6). There were no significant differences (P>0.05) in final length between treatments. However, there is a trend towards decreasing survival with decreasing salinity. The survival rate of fish cultured at 15‰ was significantly lower (P<0.05) than that of the fish cultured at 35‰.

**Table 4.3.5. Final length and survival of *R. tapirina* juveniles cultured for 30 days in water of different salinities ($\bar{x} \pm$ s.e., n=3 replicates).**

| Salinity (‰) | Final length (mm) | Survival (%) |
|---|---|---|
| 35 | 14.9 (0.2) | 100 (0) [a] |
| 25 | 14.7 (0.2) | 94.7 (2) [ab] |
| 15 | 14.8 (0.2) | 91.3 (1) [b] |

Figures in the same column sharing a common superscript are not significantly different ( P>0.05).

The data did not show homogeneity of variance due to the high survival in all replicates at 35‰ and could not be transformed, so the ANOVA result is an approximation and with only 95‰ significance may not be important.

**Table 4.3.6. Results of one-way ANOVA comparing the final lengths, and survival (arc sine $\sqrt{}$ transformed), of *R. tapirina* larvae cultured at different salinities.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 0.078 | 0.039 | 0.400 | **P > 0.05** |
| Error | 6 | 0.582 | 0.097 | | |
| Total | 8 | 0.660 | | | |
| Survival | | | | | |
| Model | 2 | 0.135 | 0.068 | 6.076 | **P < 0.05** |
| Error | 6 | 0.067 | 0.011 | | |
| Total | 8 | 0.202 | | | |

## 4.4. DISCUSSION

*Systems*

In the present study a tank volume of 200 l (160 l of water) was chosen for mass-rearing and 3 l for experimental studies. Both tank volumes were successful as rearing tanks, but the larger volumes were easier to maintain and proved suitable for the production of large numbers of metamorphosed juveniles.

Many and varied culture systems have been used for experimental or commercial culture of marine fish larvae (Table 4.4.1.). In fish that do not inflate a swim bladder, such as the greenback flounder (*Rhombosolea tapirina*), a small tank is adequate as a rearing vessel. It is easier to manipulate the environment in small tanks or systems with small water volumes and it is also possible to maintain large numbers of individual tanks. Having a large number of individual tanks in the same system allows for the replication necessary to obtain statistically significant results. It is also possible to provide a greater number of treatments to give a greater range of possible outcomes. In situations where space and/or seawater availability is limited, small-scale recirculation systems are also advantageous. It is generally possible to use the results from small-scale experiments to design larger and more commercially viable systems.

Many of the early studies on fish larvae were carried out in small containers (Flüchter, 1972a; Shelbourne, 1975; Goodfellow *et al.*, 1985; Devauchelle *et al.*, 1986; Russel and O'Brien, 1988). Some authors have found no difference between small and large tanks as rearing vessels for marine fish larvae (Howell, 1979; Devauchelle *et al.*, 1986; Person Le Ruyet *et al.*, 1981). However, the trend has been towards larger rearing tanks to allow greater water volume to wall and floor area, and a consequent reduction in larval contact with the tank walls (Jones *et al.*, 1974). Larger tanks also permit easier removal of the oily surface films associated with additions of live food. Surface oil films have been linked to abnormal swim-bladder inflation in fish which develop a swim-bladder at first-feed (Chatain and Ounais-Guschemann, 1990). Jones *et al.* (1974) experimented with 3,800 l tanks but found it difficult to maintain the high feed densities necessary for larval survival.

**Table 4.4.1. Stocking densities, flow rates, tank designs and lighting protocols related to survival rates, for some systems used in the culture of marine fish larvae.**

| Species | Stocking density (fish/l) and flow rate (l/hour) | Volume ($m^3$), shape* | Light intensity (lux) and photoperiod (hours light) | Survival (%) | Reference |
|---|---|---|---|---|---|
| Greenback flounder *R. tapirina* e | approx. 47, static - 45 | 0.16, H | 500-600, 24 | approx 15 | This study |
| *R. tapirina* e | 16, 20 | 0.25, C | Natural | 98 | Crawford, 1984a |
| *R. tapirina* e | 10, static | 0.25, C | Natural | 94 | Crawford, 1984a |
| Plaice *P. platessa* e | 5.5, 4 | 0.036, A | N. G. | 65.7 | Shelbourne, 1975 |
| Sole *S. solea* e | N. G. | 0.036, A | N. G. | 70 | Shelbourne, 1975 |
| Lemon sole *M. kitt* e | N. G, 2-3 | 0.036, A | 400, 12 | 32 | Howell, 1972 |
| Turbot *S. maximus* e | 30., 12 | 0.035 + 0.2, S | 2,000, 24 | 2.4-6.9 | Devauchelle et *al.*, 1986 |
| *S. maximus* e | N. G., 3.75-7.5 | 0.90, C | 1100, N. G. | 30-40 | Howell, 1979 |
| Atlantic cod *G. morhua* c | N. G. | 600,000 | Natural | 0-15 | Cachelou *et al.*, 1989 |
| *S. auratus* + *D. labrax* c | 100, N.G. | 2-20, C+H | 10-200, N. G. | 10 | Barnabé, 1990 |
| *S. maximus* c | 60, N.G. | 0.3-2, C+H | 1,000-2,000, 24 | 0-40 | Person-Le Ruyet, 1990 |
| Dolphin fish *C. hippurus* e | 50, 3.75-5 | 0.03, C | N. G., 24 | 25-50 | Ostrowski, 1989 |

e = experimental results and c = commercial production.

* C = conical, H = hemispherical, S = square, A = glass aquarium

N.G.=figures not given in the literature.

Black tanks were used in the present study as it has been shown that feeding efficiency in pelagic fish larvae during the live-prey feeding stage, is enhanced by using dark tanks and overhead lighting. Howell (1979) investigated the effect of tank colour on survival of larval *S. maximus*, using both black and white tanks and obtained significantly higher growth and survival rates using the black, rather than the white tanks. Ostrowski (1989) achieved 130% greater survival of dolphin fish (*Coryphaena*

*hippurus*) larvae in black, compared to tan coloured tanks. Dendrinos *et al.* (1984) experimented with the use of food colourings to improve the visibility of *Artemia* and observed a significant improvement in feeding efficiency using black-stained nauplii to feed the larvae of sole (*Solea solea*). However, the larvae were reared in glass aquaria. Black tanks improve the feeding efficiency of larval fish without the need for staining the food organisms. It appears that the larvae hunt visually by using the contrast of the prey against its background. The overhead light illuminates the prey organisms and makes them easily visible against the darker background of the tank walls.

*The effect of photoperiod*

The optimal photoperiod for maximum growth of first-feeding larvae of *R. tapirina* was found to be 18-24 hours light. The length of the daylight period did not significantly affect survival, as long as at least 6 hours light was provided. The same result was obtained by Fuchs (1978) with *S. solea* larvae, although no effect on either growth or survival was shown for juveniles after metamorphosis. The longer daylengths presumably allowed a longer feeding period as shown by Kiyono and Hirano (1981) for the black porgy (*Mylio macrocephalus*). Dowd and Houde (1980) showed that, with western Atlantic sea bream (*Archosargus rhomboidalis*), a photoperiod longer than 13 hours was only effective in increasing the growth rate when prey densities were low, survival was not affected. At low prey densities more time is probably required for food searching. However, in the present study, growth rates were improved with longer daylengths, even though food was not limiting.

It has been shown by Blaxter (1968; 1969) that marine fish larvae are largely visual predators. Feeding cannot take place in the dark and so, in the present study, almost complete mortality occurred by day 10 post-hatch. Starved larvae show complete mortality occurring from days 8-10 post-hatch (Section 3.3.4.). The high mortality observed in the treatment with two periods of 9 hours light per day, may have been caused by the stress associated with the increased number of abrupt changes from light to dark and vice versa. The same effect was shown for striped trumpeter (*Latris lineata*) (W. Hutchinson, Pers. comm.).

The high light intensity used during the present experiment may have contributed to the high mortality shown during the first 10 day period. Barahona-Fernandes (1979) found that there was an interaction between light intensity and photoperiod that further complicated the effects. Under a constant photoperiod from hatch, growth of sea bass (*Dicentrarchus labrax*) was best at 18 hours light, but survival was better at 12 hours light. Better growth, but poorer survival, was obtained at higher light intensities using

light intensities of 150-3,500 lux. Barahona-fernandes (1979) also found that, if the light intensity was lowered during the first week, then continuous lighting gave better survival and a 14-16 hour photoperiod gave better growth. However, the higher growth rates may have been due to the lower stocking densities resulting from increased mortality.

Low light intensities appear to be important for growth during the yolk sack stage of some species but this has not been documented with the Pleuronectiformes. During the present study, an initial experiment was carried out with yolk-sac larvae in 3 l tanks with three batches kept in the dark and three in the light. All larvae kept in the dark were dead before the yolk-sac had been absorbed. This was possibly due to the larvae becoming inactive when no light was present and sinking to the tank base with resultant mortality, as found by Jones (1972) with *S. maximus*. In an investigation into the effect of light intensity on the larvae of black porgy (*M. macrocephalus*), Kiyono and Hirano (1981) found that a light intensity of 3,000 lux gave the best survival and growth of larvae, and growth of postlarvae, but not of juveniles. Feeding was almost continuous during periods of light but ceased in the dark so that 24 hour lighting resulted in increased growth rates. It is not clear whether the yolk-sac larvae were subjected to the same light intensities. Ounais-Guschemann (1989) found that the growth rate of larval sea bream (*Sparus auratus*) was faster in light intensities of 600-1,300 lux than in 150-300 lux. In the present study, light intensities of 300-1,699 lux were able to induce and maintain the feeding response in *R. tapirina* as shown by continued growth in all systems at the various light intensities provided.

*The effect of temperature*

The growth rate of *R. tapirina* was considerably improved by increasing the temperature up to 18-20°C. Newell (1961) and Edwards (1979) showed that seawater temperatures off the Tasmanian east coast average 17-19°C in January and February. It appears that the larvae of *R. tapirina* are adapted to these temperatures. A temperature range of 18-20°C is also optimal for *S. maximus* (Person-Le Ruyet, 1981). Fonds (1979) investigated growth rates of *S. solea* at varying temperatures and found that development at 22°C was three times as fast as at 10°C. It has been shown for *D. labrax*, that the food intake increases positively with temperature. Increased feeding rate is sufficient to cover the increased energy demand at the higher metabolic rate (Ronzani-Cerqueira, 1991).

No significant effects on survival rate were observed in the present study, but this is probably due to the fact that the temperatures used were within the tolerance range of

the species. The survival rate of *D. labrax* larvae was found to decrease at high water temperatures around 25°C (Ronzani-Cerqueira, 1991).

*The effect of salinity*

Salinity had no effect on growth rate in the present study but the survival rate was reduced at a salinity of 15‰. This salinity also had a detrimental affect on eggs (Chapter 3). It appears that *R. tapirina* is not tolerant of salinities much below 25‰. However, in laboratory studies, *R. tapirina* juveniles showed a preference for water of 5‰ when placed in a salinity gradient (Crawford, 1984b). Thomas (1993) found that there was a significant decrease in blood plasma osmolality of *R. tapirina*, commencing after three days exposure to water of 10‰ salinity, which was not recorded at either 25 or 35‰. The mortality rate was also higher. The effect of salinity on 0-group European flounder (*Platichthys flesus*) was studied by Hutchinson and Hawkins (1990). These authors found that there was little difference in the blood osmolality between fish in high and low salinities, but that osmoregulation was more difficult in fluctuating salinities, such as those encountered in natural estuarine habitats. Water loss and gain was controlled by altering the permeability of the gills through changes in blood flow and ventilation rate. This suggests that there should have been no differences in survival at the constant salinities used in the present study. In a study of thirteen marine fish species by Wu and Woo (1983), it was found that the majority of species could survive for two weeks at salinities down to 5 - 10‰. There appeared to be a critical tolerance limit for each species below which osmoregulatory failure occurs. Inshore species were found to be more euryhaline than offshore species. However, this study was carried out with adult fish and the tolerance range of metamorphosing juveniles might be expected to be narrower due to a less well developed osmoregulatory system.

The results of the present study show that:

1. The larvae of *R. tapirina* can be cultured successfully in black hemispherical tanks of 3-160 l capacity.

2. The larvae of *R. tapirina* grow faster under 18-24 hour light conditions and has no effect on the survival rate.

3. High light intensities of 1,699 lux during the yolk-sac stage may cause increased mortality of *R. tapirina* larvae, but growth and feeding occur with light intensities of 300-1,699 lux.

4. Temperatures of 18-20°C improve the growth rate of *R. tapirina* larvae with no effect on the survival rate.

5. Salinities of below 25‰ can cause increased mortality of *R. tapirina* larvae, but have no effect on the growth rate over sustained periods; this is an area requiring more research.

# CHAPTER 5

# LARVAL DEVELOPMENT AND FEEDING

## 5.1. INTRODUCTION

The feeding of marine fish larvae has received a great deal of attention over the past twenty years and is still considered to be a major barrier to rapid growth of the industry. However, major advances have been made recently in our understanding of: digestive tract development, enzyme formation, and nutritional quality of live feeds.

There have been many recent studies into the morphological development of the digestive tract of marine fish larvae (Cousin and Baudin Laurencin, 1985; Kjørsvik *et al.*, 1991; Boulhic and Gabaudan, 1992; Walford and Lam, 1993). The main aims of these studies were to determine the age at which the digestive system is capable of digesting complex compounds, such as artificial feeds, and to increase our understanding of the qualitative and quantitative nutritional requirements of the larvae. Segner *et al.* (1993) proposed that a functional stomach capable of secreting pepsin, is required for the digestion of dry feeds, although the reasons for this are not fully understood.

The digestive enzymes of fish larvae have also been the subject of numerous studies, as it is thought that the primitive enzyme systems of larval fish are not sufficiently developed to digest artificial diets (Tanaka *et al.*, 1972; Lauff and Hofer, 1984; Clark *et al.*, 1986; Clark *et al.*, 1987; Uys *et al.*, 1987; Hjelmland *et al.*, 1988; Munilla-Moran and Stark, 1989; Munilla-Moran *et al.*, 1990; Hjelmland *et al.*, 1993). The digestive capacity of the larvae appears to increase with the consumption of live feeds at first-feeding (Munilla-Moran and Stark, 1989). Munilla-Moran *et al.* (1990) proposed the theory that, although the larvae have a limited digestive capacity at first-feeding, based on tryptic activity, they rely on the digestive enzymes from the live feeds to increase their digestive ability. These authors found that the digestive enzymes of the rotifers and *Artemia* also increase if they are fed before distribution to the fish larvae.

Attempts to include exogenous enzymes in microdiets fed to larval sea bream (*Sparus auratus*) have led to slightly increased growth rates, but these are still not as good as those achieved using live prey (Kolkovsky *et al.*, 1990). The bacterial composition of the larval gut may also be important in improving its digestive capacity, and is influenced by the bacterial composition of the water in which the live prey is cultured (Blanch *et al.*, 1991; Munro and Birkbeck, 1993; Munro *et al.*, 1993; Skjermo and Vadstein, 1993).

May (1970) reviewed the literature on larval fish feeding and documented a number of feed species that have been used successfully. However, for commercial culture there

are generally only two species that are used. These are the marine rotifer (*Brachionus plicatilis*) and the brine shrimp (*Artemia* sp.) in its juvenile stages. Marine copepods are used occasionally but although they have a high nutritive value (Watanabe *et al.*, 1980), they are difficult to rear at sufficient densities for commercial culture (Tucker, 1992).

The most important criteria involved in the selection of feeds for marine fish larvae are: availability, density, appearance, size, possibly smell or taste, nutritional quality, digestibility and stability (Tucker, 1992). The size of the prey that can be successfully ingested, depends on the gape size of the fish larvae. Hunter (1984) suggested that optimal prey sizes for first-feeding marine fish larvae were approximately 25% of the width of the mouth, increasing to 50% of the mouth width thereafter. Shirota (1970) developed a formula for the calculation of gape sizes for marine fish larvae, based on the length of the upper mandible, assuming a maximum mouth opening angle of 90°. This formula can be used to predict the size of prey that should be offered to the larvae at any stage during their development.

Due to the small size of larval marine fish at first-feeding, the rotifer is an ideal initial food organism. The use of rotifers has been reviewed by Lubzens *et al.* (1989). The rotifer is relatively simple to culture at high densities and its slow swimming speed renders it easily caught and ingested by early larval fish. However, the nutritional value of rotifers is variable and cultures are often unstable with frequent, sudden population declines (Fulks and Main, 1991).

The Brine shrimp (*Artemia* sp.) has long been used in the rearing of marine flatfish. It is readily available and has a long storage time in the form of cysts which are easily decapsulated using the method described by Sorgeloos *et al.* (1977). However, due to its large size, when compared to the rotifer, its use as a first-feed is restricted to species with large larvae, such as plaice (*Pleuronectes platessa*) (Shelbourne, 1964) and sole (*S. solea* ) (Person-Le Ruyet, 1990).

The feeding regimes used for intensive marine fish larviculture, usually involve rotifers for a short early period of 1-2 weeks, followed by Instar I *Artemia* nauplii for a few days and finally, Instar II nauplii until the larvae are able to be weaned onto a dry feed. It is possible to delay the initial feeding of sea bass (*Dicentrarchus labrax*) until day 10 post-hatch if the larvae are kept in the dark or at reduced salinities (Johnson and Katavik, 1986; Barnabé, 1990; Sweetman, 1992). This results in larger larvae that are able to ingest *Artemia* nauplii at first feeding and therefore, reduces the reliance on rotifers and consequently, the cost of production. The feeding of fish larvae has been reviewed by May (1970), Dabrowski (1984) and Tucker (1992).

The nutritional value of *Artemia* was reviewed by Léger *et al.* (1987) and it was concluded to be highly variable. This variability can be avoided by using high quality strains, such as San Francisco Bay, Brazilian or Reference strain, when using freshly hatched instar I nauplii. Klein-Macphee *et al.*, (1982) found that the Australian strain was also of high nutritional value. Decapsulation of cysts can be beneficial in avoiding bacterial contamination. Poor digestibility and inadequate nutritional quality have been reported for instar I *Artemia* nauplii (Watanabe *et al.*, 1978; Katavic, 1986; Sorgeloos *et al.*, 1988). The application of enrichment techniques when using instar II *Artemia* nauplii, is essential (Léger *et al.*, 1987).

The latest improvements in marine fish larviculture are discussed by Sorgeloos and Léger (1992). Important advances have been made recently in the areas of nutrition, and management. The nutritional value of live organisms was reviewed by Watanabe *et al.* (1980) and it was concluded that the best feeds were those that contained high levels of the n-3 highly unsaturated fatty acids (HUFA), eicosapentaenoic acid (EPA; 20: 5n-3) and docosahexaenoic acid (DHA; 22: 6n-3). Feeds that were poor in these essential fatty acids (EFA) could be improved by the use of enrichment techniques. Watanabe (1991) suggested that it is the DHA content of the live feeds that has the major influence on the performance of marine fish larvae. Marine fish eggs have a high DHA content which decreases rapidly at hatching. DHA may be used either as an energy source or converted to other physiologically important compounds such as prostaglandins (Watanabe, 1991; 1993).

There appears to be a connection between the nutritional quality of the live feeds and malpigmentation in flatfish, although this may not be the only cause. Problems with the pigmentation of the ocular surface of cultured flatfish have been recorded by a number of authors (Seikai *et al.*, 1987a; Grønas *et al.*, 1993; Kanazawa, 1991; Kanazawa, 1993). There may also be a link between abnormally high HUFA levels, or bacterial infection, and malpigmentation (Grønas *et al.*, 1993). Fukusho *et al.* (1986) found that high aeration had no effect on the degree of pigmentation, even though higher aeration rates resulted in severe physical disturbance of the larvae. This may be of questionable significance because the authors used 20 day old larvae for the trial, and the problem of malpigmentation appears to arise during the early period of larval development (Fukusho *et al.*, 1987). Nakano *et al.* (1992) implicated zinc and copper deficiencies in malpigmentation and Fukusho *et al.* (1987) showed that the rate of malpigmentation in Japanese flounder (*Paralichthys olivaceus*) could be reduced by feeding larvae with the eggs of red sea bream (*Pagrus auratus*) when the larvae reached 8.5-9.0 mm. Sugiyama and Yano (1989) found that malpigmentation could be increased by the addition of thiourea ($SC(NH_2)_2$) to the diet and concluded that the

phenomenon was caused by the inhibiting effects of thiourea on tyrosinase and/or thyroid function. No reference to malpigmentation of the blind side could be found in the literature.

The specific objectives of the present study were:

1. To determine the gape sizes of *R. tapirina* larvae during development from first-feeding to metamorphosis, so that the gape size and therefore the required feed particle size, could be estimated and related to fish length.

2. To document the behaviour and external appearance of *R. tapirina* larvae from first-feeding to metamorphosis, in order that future workers can recognise the normal stages of development.

3. To examine the development of the gut of *R. tapirina* larvae in order to relate gut development to fish length and gape size, and predict the length and age at which artificial feeds could be digested.

3. To assess the performance of various enrichment diets for live feeds, in terms of their effect on growth, survival and pigmentation of the larvae of *R. tapirina.*

## 5.2. MATERIALS AND METHODS

### 5.2.1. Larval development and behaviour

A batch of *R. tapirina* larvae was cultured to metamorphosis in order to document the timing of changes in behaviour and pigmentation during the larval stages. Approximately 7,500 flounder eggs (47/l) were stocked in a 160 l black fibreglass hemispherical tank in a recirculating system (Appendix 1.3). Temperature was maintained at 15.5-16°C and a continuous light regime was used, giving a light intensity at the water surface of 500-600 Lux. The larvae were fed twice daily from day 4 post-hatch, with rotifers enriched with microalgae (Appendix 1.2.) at a density of 10/ml. Instar II nauplii of *Artemia* were added at a rate of 1/ml together with the rotifers, at day 10 post-hatch. Rotifers were discontinued after day 14 as all larvae were observed to contain *Artemia* in the gut by this time. *Artemia* were increased to 2/ml at day 17 and 3/ml at day 20. A static system was employed for the rotifer feeding stage. Uneaten rotifers were flushed from the tank by turning on the water flow (180 l/hour) and using a 500 µm screen on the outlet, for 2-3 hours daily, before the addition of newly-enriched live feed.

During the *Artemia* feeding stage a water flow rate of 45 l/hour was used and a 125 µm screen was used on the outlet. Uneaten *Artemia* were flushed out of the tank by changing this for a 500 µm screen for 2-3 hours daily. A random sample of ten larvae was removed every second day, and the stage of development recorded, as well as the behaviour and external colouration of the larvae remaining in the tank.

### 5.2.2. The development of the gut

The development of the digestive tract was studied using standard histological techniques and related to the external appearance of the larvae. A batch of larvae was cultured under normal conditions (160 l tank, 24 hour light, 500-700 lux, 15-16°C) and fed on rotifers and *Artemia* enriched with microalgae (Appendix 1.2). Random samples of 10 larvae were taken daily from day 0 up to day 30. Larvae for histological examination were placed live in 10% phosphate buffered formalin. Live larvae were photographed after anaesthetisation in 20 ppm benzocaine.

Larvae for histological examination were prepared by clearing in xylene, fixing in alcohol, embedding in paraffin wax and staining with standard haematoxylin-eosin staining techniques. Sections were cut approximately in the sagittal plane. As the larvae were not straightened prior to sectioning the sections were not always completely accurate, particularly for the smaller larvae.

Photographs of the anaesthetised or sectioned larvae were taken using a Wild M37 dissecting microscope with a Wild MPS46 photoautomat and a Wild MPS52 camera using T120S tungsten film.

### 5.2.3. Gape size and feeding regime

In order to determine the time at which *Artemia* could be ingested by *R. tapirina* larvae, the increase in gape size was measured for the period of larval rearing and related to the larval length and age. These larvae were from the same batch used in section 5.2.1. above and were therefore, cultured under the conditions as described in that section. A random sample of ten larvae were measured (notochord length until formation of the caudal fin and total length thereafter) every second day until metamorphosis (day 31), at which time a sample of thirty fish was measured and weighed. A measurement of the lower jaw was taken at the same time as length measurements throughout the experiment. This measurement was assumed to be the

same as for the upper jaw but was easier to measure due to its prominence in *R. tapirina*. The measurement was transformed using the equation:

$D = \sqrt{2} \times$ length of lower jaw (adapted from Shirota, 1970)

Where D= gape size or height of the mouth opening (mm).

### 5.2.4. The effect of different enrichment diets

A number of experiments were carried out to investigate the effectiveness of various commercial enrichment products for improving the nutritional quality of the live feeds used for larval rearing. Nutritional quality was measured by improvements in growth and pigmentation.

#### (i) Experiment 1.

A set of nine, black hemispherical plastic 3 l tanks was arranged to give three treatments and three replicates (Section 4.2.1.). Flow rates were maintained at 8 l/hour per tank. Each set of three tanks was separated and filtered through an individual filter system. Temperature was maintained at 15-16°C and continuous light was provided by overhead fluorescent tubes at an intensity of 300-400 lux. Fifty flounder larvae at 17 days post-hatch (initial length = 4.54 ± 0.06 mm; $\bar{x} \pm$ s.e., n=50 larvae) were added randomly to each tank. All fish were from the same brood and had previously been fed on rotifers enriched with microalgae (Appendix 1.2.). Throughout the experiment feeding was carried out twice daily.

Feeding rates were 10 rotifers/ml up to day 19 (day 2 of the experiment). A mixture of 1 *Artemia* /ml and 5 rotifers/ml were fed up to day 23 (day 6 of the experiment) After which the *Artemia* were increased to 2/ml and rotifers were discontinued. From day 31 post-hatch (day 13 of the experiment) *Artemia* were fed at a rate of 4/ml.

All fish were weighed and measured (total length, Appendix 1.4.) at day 60 post-hatch (day 43 of the experiment). Survival was calculated from the number of surviving fish and condition (K) was calculated using the formula:

$$K = \frac{\text{weight} \times 100}{\text{length}^3}$$

*Enrichment*

Enrichment was carried out for 16-18 hours, after which half the enriched animals were distributed to the fish and the remainder were left for a further 6-7 hours, with a second addition of the enrichment diet, prior to distribution. Enrichment took place in 10 l of water in a 20 l bucket with strong aeration. Three different enrichment diets were used:

Diet 1: 'Frippak booster' (Sanofi Aquaculture, Paris, France) applied at a rate of 0.5 g/$10^6$ rotifers/day and 1g/$10^6$ *Artemia* /day.

Diet 2: A mixture of the microalgae *Isochrisis* sp. (Tahitian clone) and *Pavlova lutheri* fed to excess (there was always algae remaining in the enrichment bucket when rotifers or *Artemia* were harvested for distribution to the larvae).

Diet 3: 'Diet A', a mixture of dried *Spirulina* (37%), brewers yeast (37%), cod liver oil (20%), vitamins (4%) and choline chloride (2%) (Robin *et al.*, 1984; M. Daintith pers. comm.), applied at a rate of 1 g/$10^6$ animals/day.

**(ii) Experiment 2**

This experiment was conducted at the same time as Experiment 1 with fish from the same brood but was unreplicated. A set of three 160 l black, hemispherical fibreglass tanks in a recirculating system (Appendix 1.3.) were each stocked with approximately 10,000 newly-fertilised flounder eggs. The water flow rate was set at 45 l/hour after the first week, prior to which the tanks were left static. Continuous light was provided by overhead fluorescent tubes at an intensity of 500-700 lux. At day 4 post-hatch, feeding commenced and was carried out as in Experiment 1. Enrichment also was as in Experiment 1, with a different diet used for each tank. Samples of fish were taken and measured for total length at days 13, 34 and 62 post-hatch. In the last sample, the number of malpigmented fish was also recorded. Survival was not recorded as mortalities were too difficult to detect in these tanks and the initial number of eggs was not known accurately.

**(iii) Experiment 3**

A similar trial to Experiment 2 above, was carried out in 1992 and using different enrichment regimes as follows:

Diet 1: 'Frippak booster' at a rate of 0.5 g/$10^6$ rotifers/day and 1g/$10^6$ *Artemia* /day.

Diet 2: A mixture of the microalgae *Isochrisis* sp. (Tahitian clone) and *Pavlova lutheri* fed to excess (there was always algae remaining in the enrichment bucket when rotifers or *Artemia* were harvested for distribution to the larvae).

Diet 3: As for Diet 2 to day 15 followed by Diet 1 until the end of the experiment.

Enrichment was carried out at a temperature of 21-22°C but was otherwise the same as in Experiments 1 and 2 (Appendix 1.2.).

Approximately 7,500 *R. tapirina* larvae (initial length = 2.98 ± 0.04 mm; $\bar{x}$ ± s.e., n=10 larvae) were stocked in each 160 l tank at day 4 post-hatch and feeding commenced immediately. Feeding rates were 10 rotifers/ml to day 12 followed by a mixture of 1 *Artemia* /ml and 5 rotifers/ml to day 14 post-hatch. *Artemia* only were fed at a rate of 2/ml from day 14 post-hatch until day 31 when the experiment was terminated, as metamorphosis was complete in all tanks. These fish were then weaned separately to determine the effect of the larval diet on weaning survival (Section 6.3.5. (i)). Measurements of total length (Appendix 1.4.) were made at days 4, 10, 16, 24 and 31 post-hatch. A random sample of thirty fish from each tank was weighed and measured on day 31 post-hatch. The percentage of malpigmented fish in each tank was also determined at this stage, by counting the numbers in a sub-sample of 250 fish per treatment.

**(iii) Experiment 4**

This trial was similar to Experiment 3 but undertaken in 1993 and using different enrichment regimes as follows:

Diet 1: 'Frippak booster' applied at a rate of 0.5 g/$10^6$ rotifers/day and 1g/$10^6$ *Artemia* /day for a 24 hour enrichment period.

Diet 2: Nutri-Pack (an emulsion from Gulf Pacific Industries Ltd, Albany, Auckland, New Zealand) applied at a rate of 0.25 ml/l for rotifers over a 2 hour period and 0.25 ml/l for *Artemia* over a 24 hour period.

Diet 3: A mixture of the microalgae *Isochrisis* sp. (Tahitian clone) and *Pavlova lutheri* fed to excess for 24 hours (there was always algae remaining in the enrichment bucket when rotifers or *Artemia* were distributed), to day 15 followed by Diet 1 until the end of the experiment.

Approximately 10,000 fish (initial length = 2.81± 0.05 mm; $\bar{x}$ ± s.e., n=40 larvae) were stocked in each tank at day 0 post-hatch and rotifers were added from day 3 post-hatch. Feeding rates were 10-20 rotifers/ml fed once daily, to day 11 and then a mixture of 1 *Artemia* /ml and 5 rotifers/ml fed once daily, to day 14. *Artemia* only, were fed once daily, at a rate of 2 /ml from day 14 post-hatch until day 31 when the experiment was terminated as metamorphosis was complete in all tanks. These fish were then weaned to determine the effect of the larval diet on weaning survival (Section 6.3.5. (ii)). Measurements of notochord or total length (after flexion) of ten fish from each tank, were made every second day. A random sample of thirty fish from each tank was weighed and measured on day 31 post-hatch. The percentage of malpigmented fish in each tank was also determined at this stage by counting the numbers in a sub-sample of 300 fish per treatment.

## 5.3. RESULTS

### 5.3.1. Larval development and behaviour

The behaviour and external colouration of larvae as related to the developmental stage is shown in Table 5.3.1. The mean length of newly-hatched larvae (stage 1, Appendix 1.5.2.) was 1.98 ± 0.03 mm, with a yolk sac length of 0.986 ± 0.01 mm and height of 0.575 ± 0.02 mm ($\bar{x}$ ± s.e., n=10 larvae). Feeding motions commenced in some larvae on day 4 post-hatch (stage 2, Appendix 1.5.2.), though it was not possible to identify rotifers in the gut until day 6 post-hatch. Feeding was observed as a shivering motion at the water surface rather than the S-shaped striking movements normally associated with fish larvae. The gut had a complete loop by day 12 post-hatch and the caudal fin rays were developing. The larvae were noticeably deeper by this stage and differed in appearance to the larvae of round fish. Flexion of the notochord (stage 3/4, Appendix 1.5.2.) had begun in all fish by day 14 post-hatch and the pigment was noticeably heavier, giving the larvae a dark appearance in the tank; eye migration had commenced in 20% of the sample and some fish were observed to be moving to the bottom of the tank. The dorsal and ventral fin rays were developing by day 16 post-hatch and many fish were observed lying on the bottom of the tank, these fish were completely dark in colour. All fin rays had completely developed by day 18. By day 20 some fish were beginning to show a lighter colouration (stage 5) due to the

formation of white pigment cells, all other fish were still dark and lying on the tank bottom or in the water column. By day 24 many fish were fully metamorphosed and had formed red and yellow pigment cells making them appear brown in colour (PLATE 2). By day 28, 90% of the fish were fully metamorphosed. Metamorphosis was complete by day 31 post-hatch.

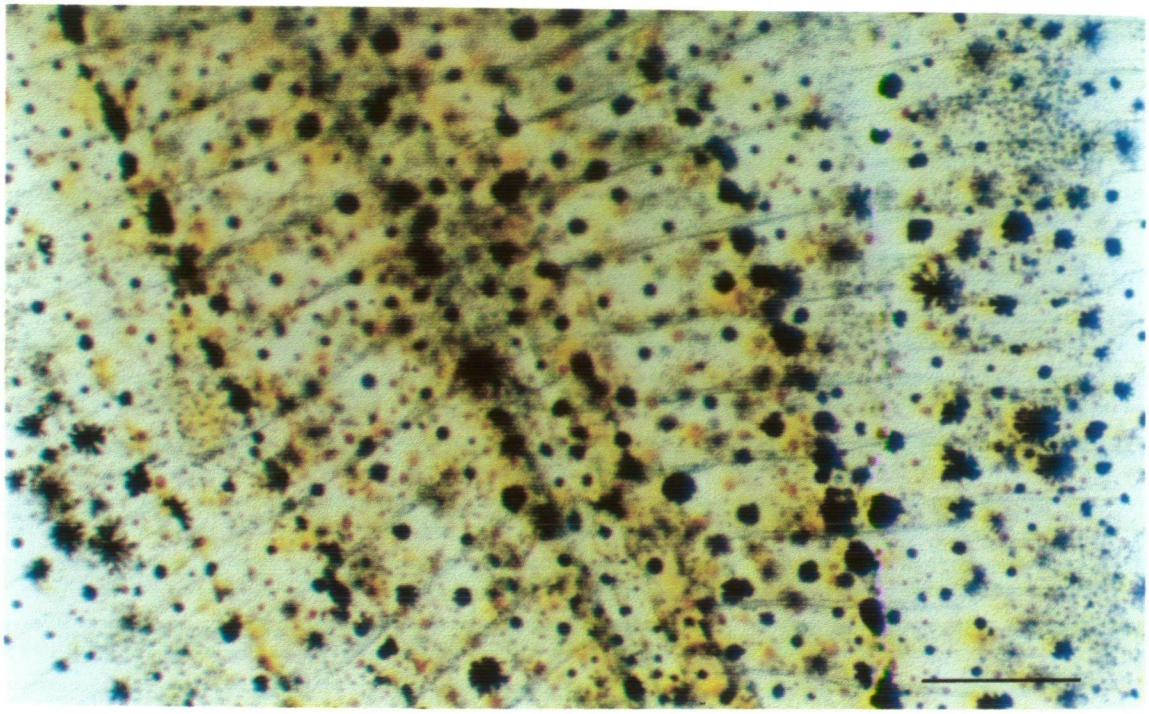

PLATE 2. Pigment cells on the ocular surface of a fully metamorphosed *R. tapirina* juvenile. Scale bar represents 200 μm.

**Table 5.3.1. Observed behaviour and external colouration of *R. tapirina* larvae up to metamorphosis.**

| Days post-hatch | Stage (Ryland, 1966, Crawford, 1986) | Position in the water column | Colour |
|---|---|---|---|
| 2 | 1 | Surface, sides | Transparent |
| 4 | 2 | Spread, around sides | " |
| 14 | 3/4 | Spread, but with some on the bottom | Some with heavy black pigmentation |
| 20 | 5 | " | Black, many with white spots |
| 22 | 5 | 50% on the bottom | " |
| 24 | 5, M | " | Many with brown colouration |
| 31 | M | Bottom | Adult colouration |

### 5.3.2. The development of the gut

At hatching (day 0 post-hatch) the larvae possessed a large yolk-sac, undeveloped eyes and jaw, and a straight gut, unopen at the anus (PLATES 3 and 4).

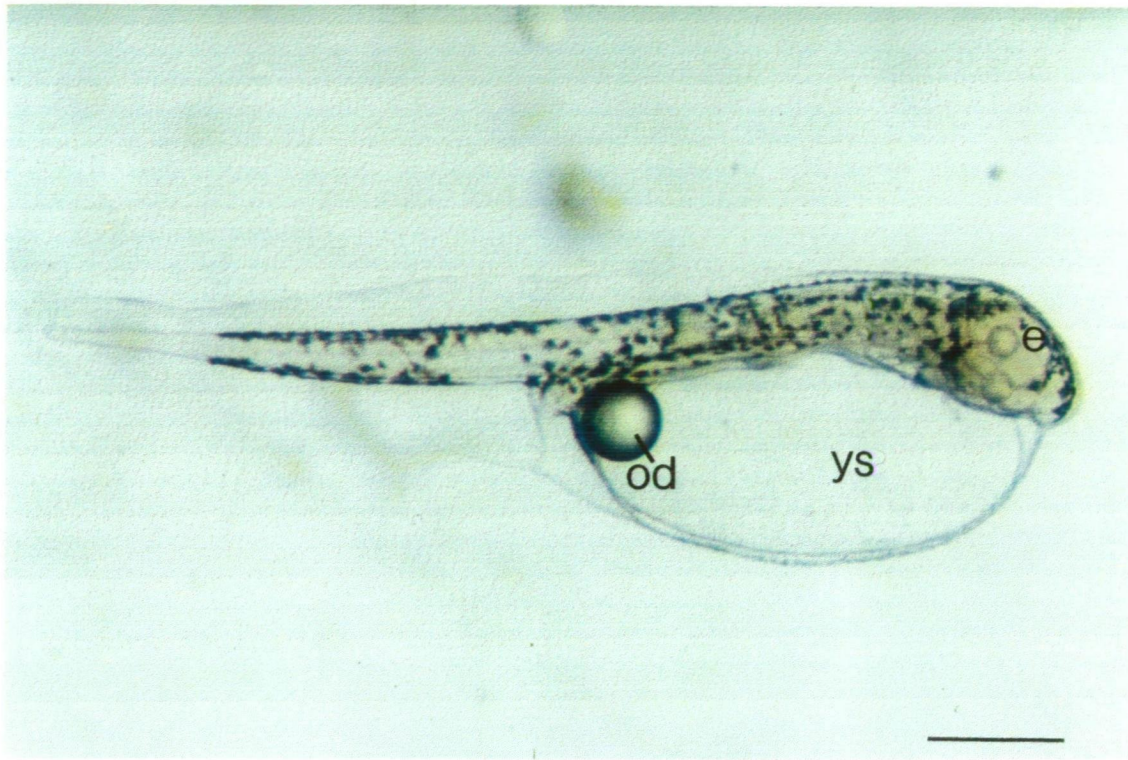


PLATE 3. External view of *R. tapirina* larva at hatching (day 0 post-hatch). ys=yolk-sac, od=oil droplet, e=eye. Scale bar represents 300 μm.

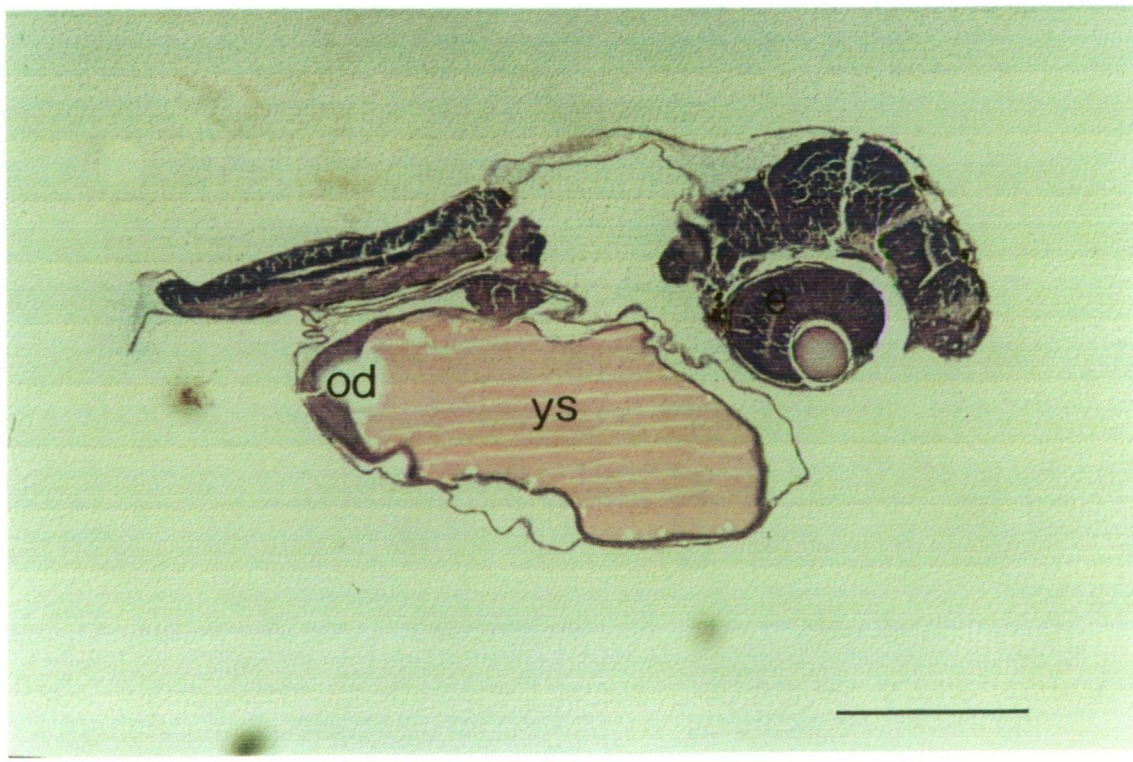


PLATE 4. Sagittal section of *R. tapirina* larva at day 0 post-hatch. ys=yolk-sac, od=oil droplet, e=eye. Scale bar represents 300 μm.

At day 1 post-hatch the gut remained straight but had further developed and become more obvious, both externally and in histological section. The bucco-pharyngeal cavity was visible in section (see PLATES 5 and 6).

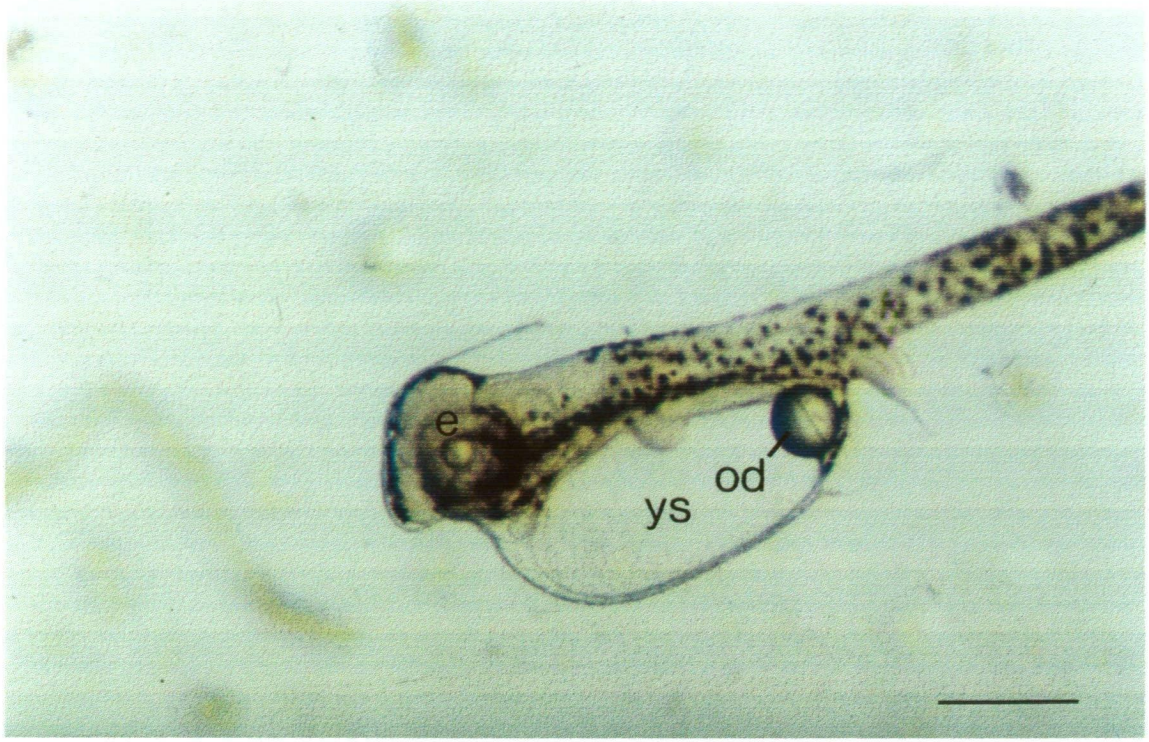


PLATE 5. External view of *R. tapirina* larva at day 1 post-hatch. ys=yolk-sac, od=oil droplet, e=eye. Scale bar represents 300 µm.

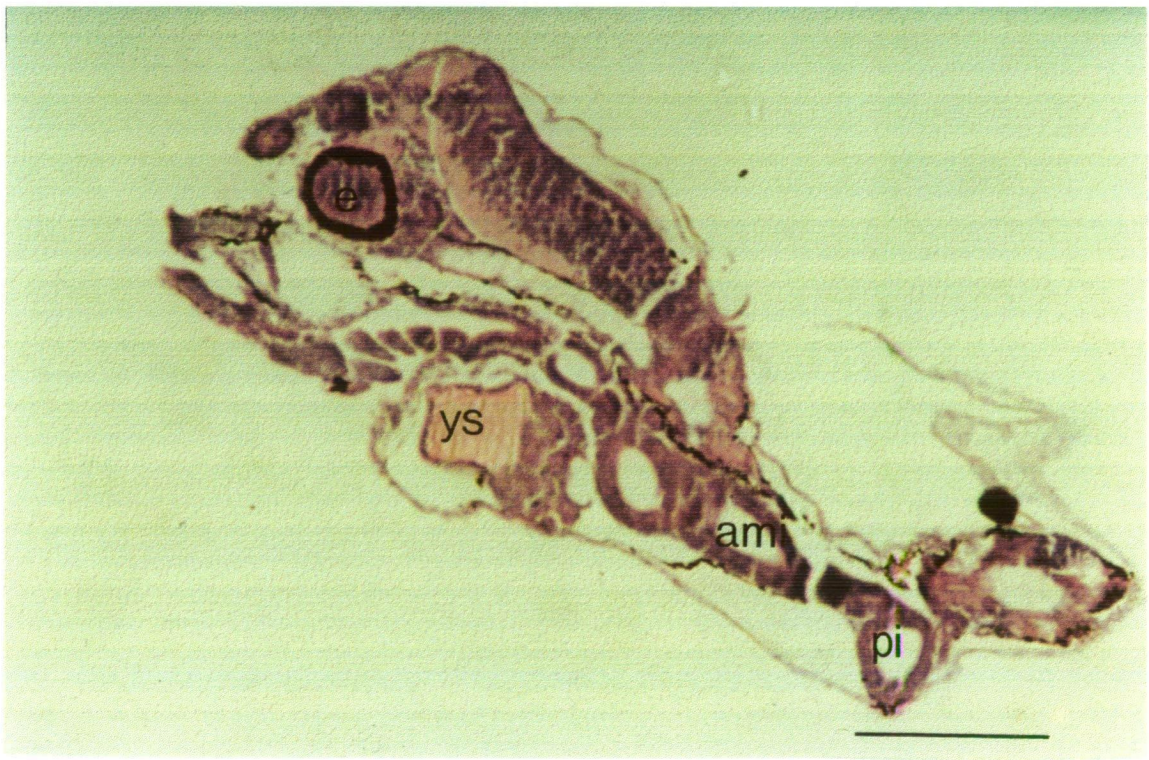


PLATE 6. Sagittal section of *R. tapirina* larva at day 1 post-hatch. ys=yolk-sac, ami=antero-median intestine, pi=posterior intestine. Scale bar represents 300 µm.

By day 2 post-hatch the yolk-sac was considerably reduced and the anterior intestine was beginning to differentiate from the remainder of the intestine. The rudimentary lower jaw could be seen externally and the eyes were pigmented (PLATES 7 and 8).

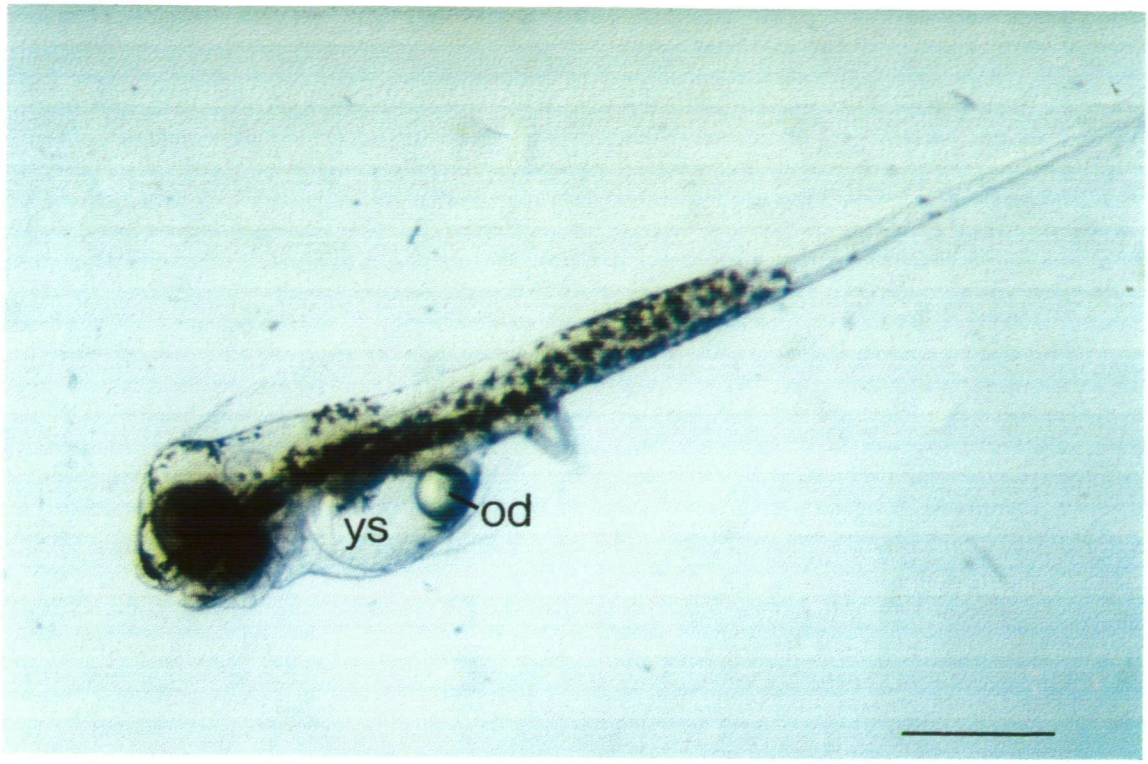


PLATE 7. External view of *R. tapirina* larva at day 2 post-hatch. ys=yolk-sac, od=oil droplet. Scale bar represents 300 μm.

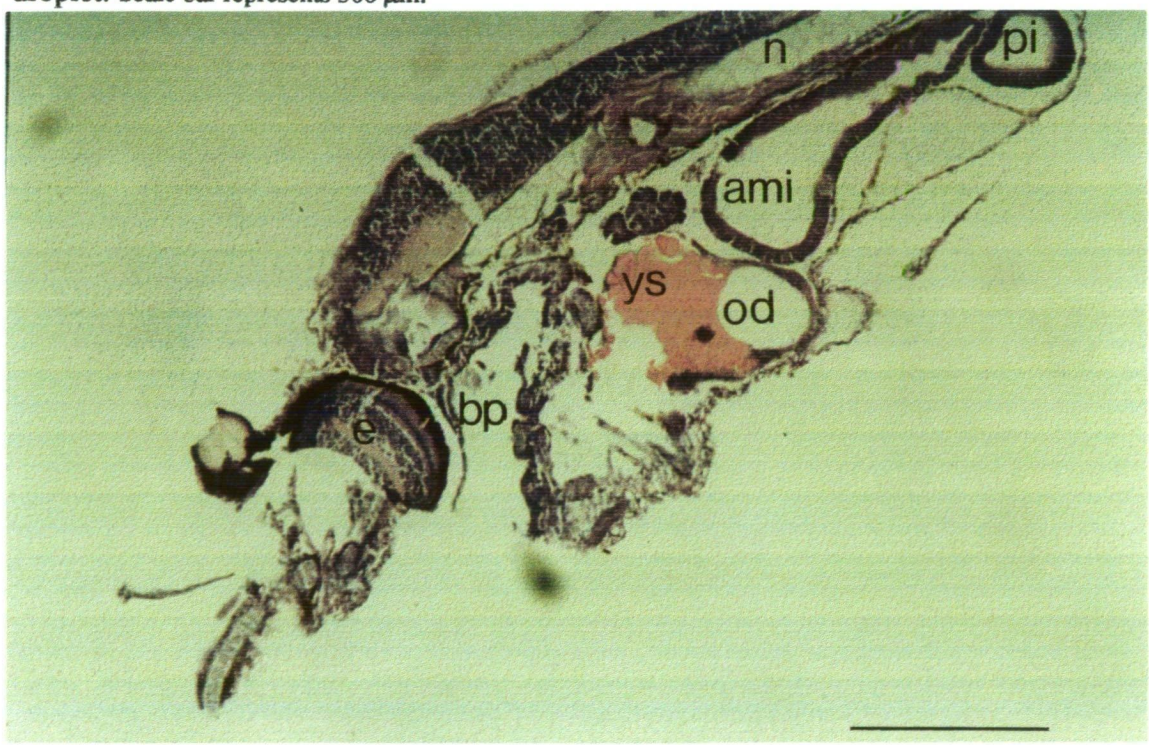


PLATE 8. Sagittal section of *R. tapirina* larva at day 2 post-hatch. ys=yolk-sac, od=oil droplet, e=eye, bp=bucco-pharyngeal cavity, n=notochord, ami=antero-median intestine, pi=posterior intestine. Scale bar represents 300 μm.

By day 3 post-hatch the anterior intestine had formed and was differentiated from the median intestine and stomach. Little yolk remained but the oil droplet was still complete (PLATES 9 and 10).

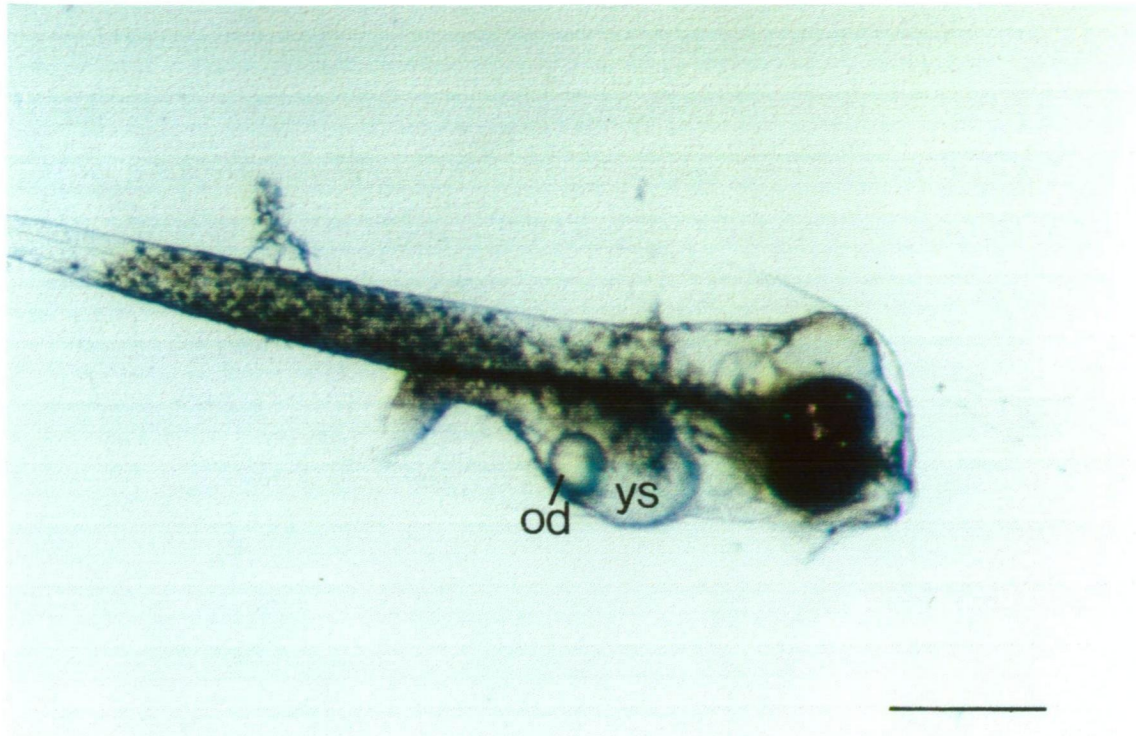


PLATE 9. External view of *R. tapirina* larva at day 3 post-hatch. ys=yolk-sac, od=oil droplet. Scale bar represents 300 µm.

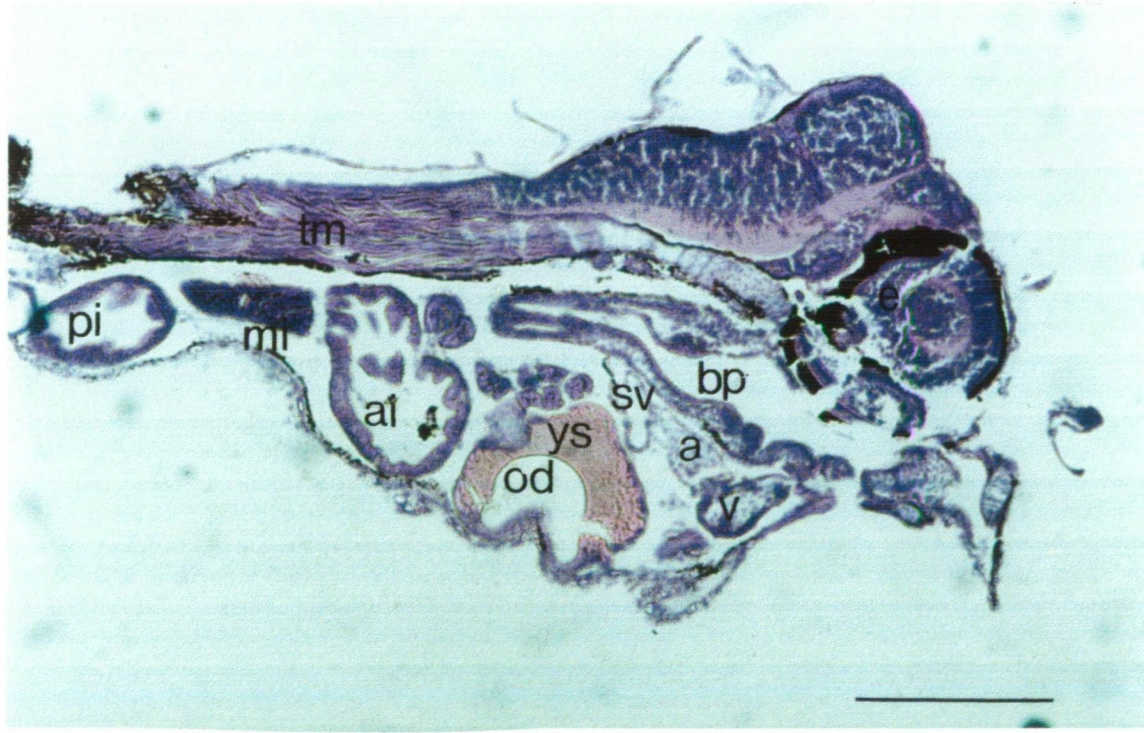


PLATE 10. Sagittal section of *R. tapirina* larva at day 3 post-hatch. ys=yolk-sac, od=oil droplet, e=eye, bp=bucco-pharyngeal cavity, pi=posterior intestine.ai=anterior intestine, mi=median intestine, sv=sinous venosus, a=atrium, v=ventricle, tm=trunk muscle. Scale bar represents 300 µm.

By day 5 post-hatch the yolk-sac and oil droplet had been absorbed and the larvae had commenced exogenous feeding (PLATES 11 and 12).

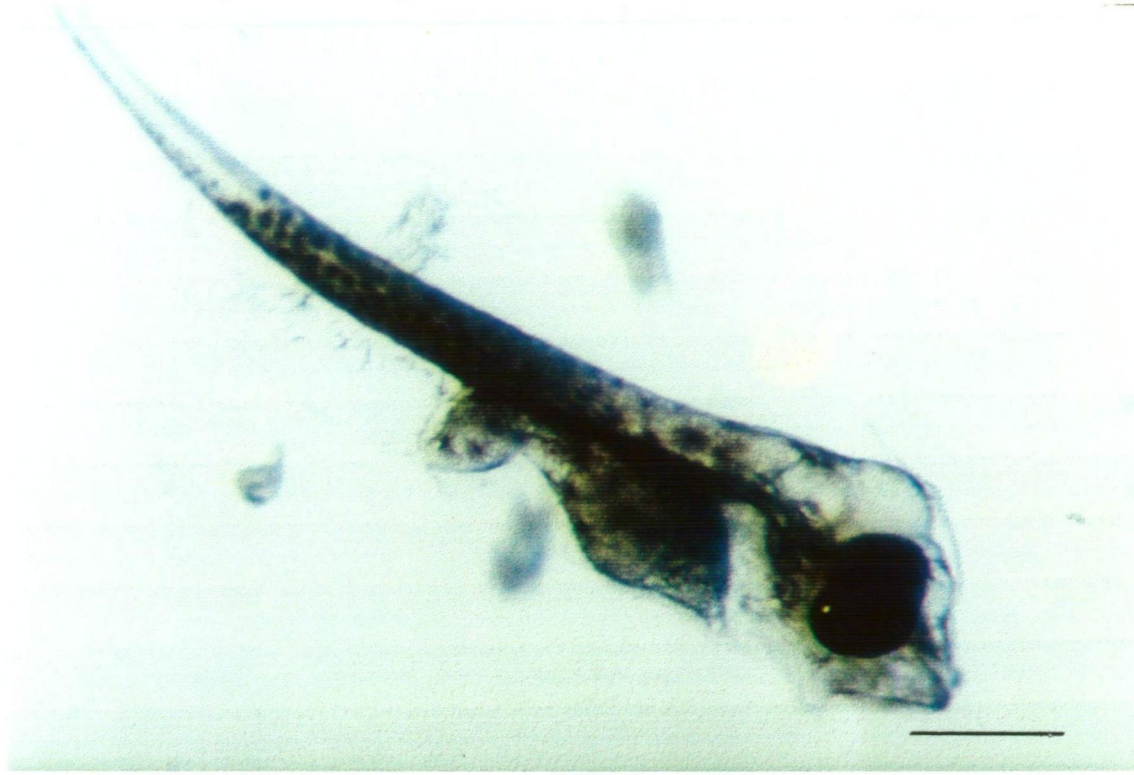

PLATE 11. External view of *R. tapirina* larva at day 5 post-hatch, the commencement of exogenous feeding. Note: the gut is full of rotifers and the jaw and eyes are fully developed. Scale bar represents 300 µm.

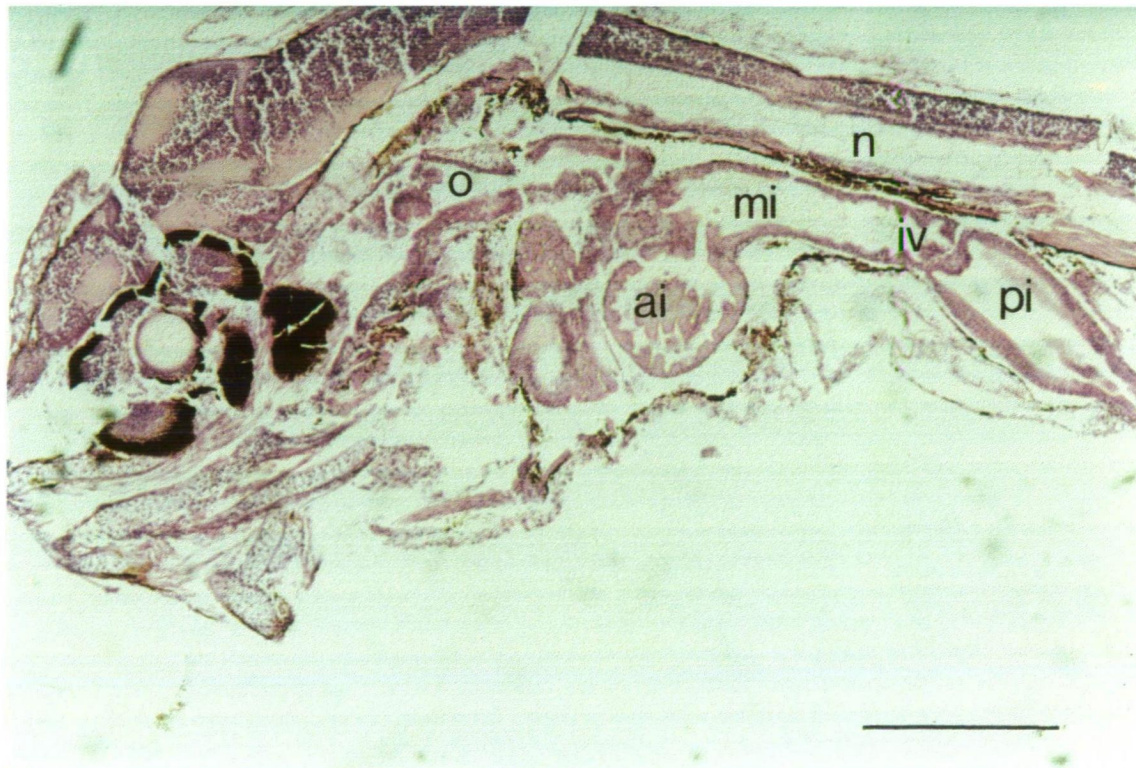


PLATE 12. Sagittal section of *R. tapirina* larva at day 5 post-hatch. n=notochord, pi=posterior intestine, mi=median intestine, ai=anterior intestine, o=oesophagus, iv=intestinal valve. Scale bar represents 300 µm.

By day 9 post-hatch the larvae were deepening dorso-ventrally as the anterior and median intestines further increased in volume (PLATES 13 and 14).

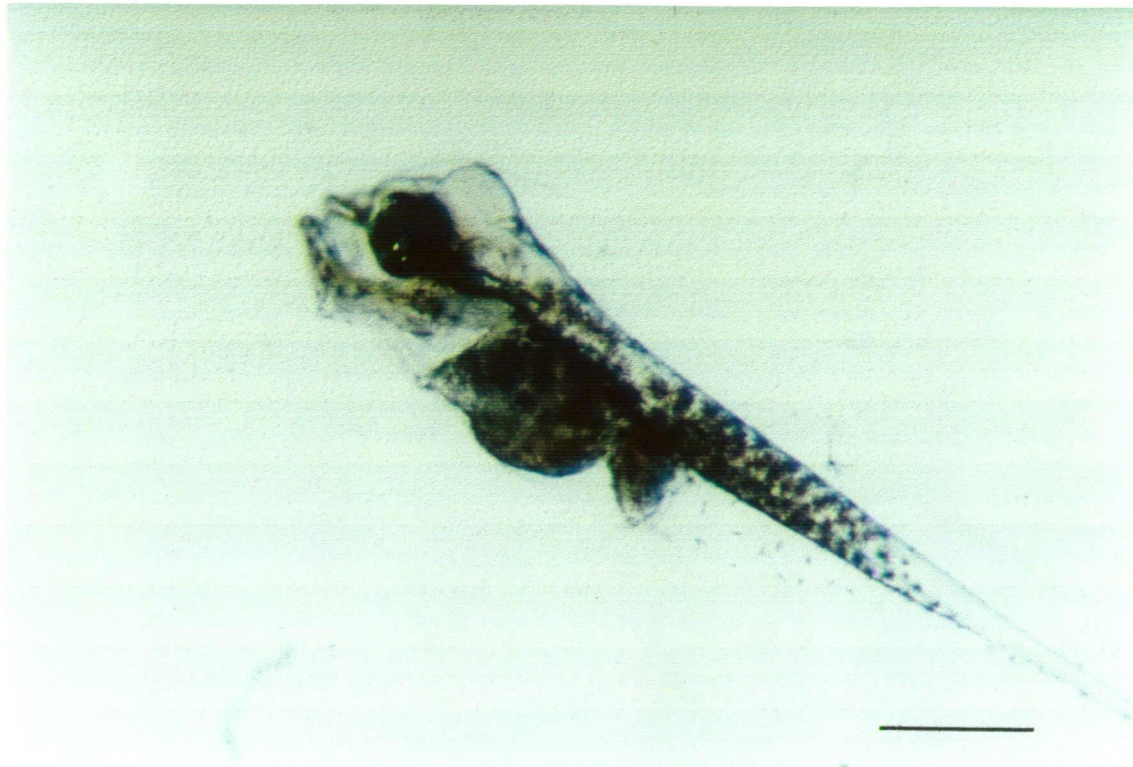

PLATE 13. External view of *R. tapirina* larva at day 9 post-hatch. Scale bar represents 500 µm

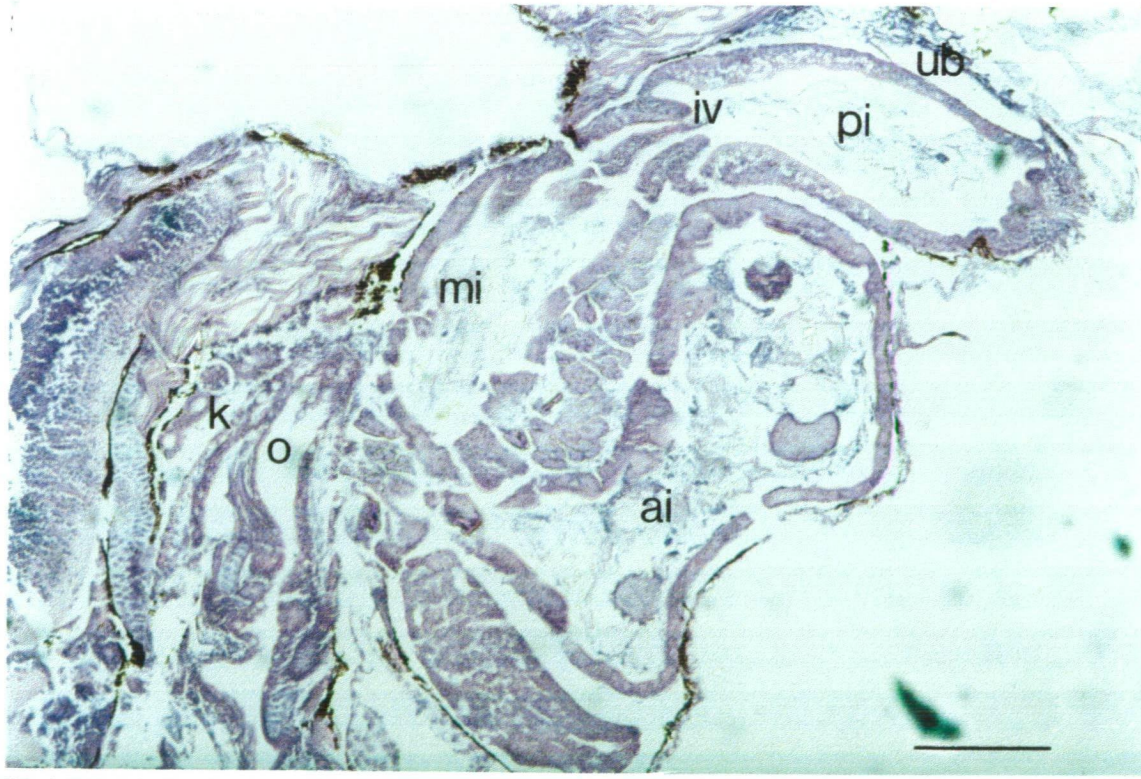


PLATE 14. Sagittal section of *R. tapirina* larva at day 9 post-hatch. Note: The stomach is hidden behind the median intestine. o=oesophagus, ai=anterior intestine, iv=intestinal valve, mi=median intestine, pi=posterior intestine.k=kidney, ub=urinary bladder. Scale bar represents 100 µm.

By day 16 post-hatch the larvae were commencing metamorphosis, taste buds were discernible and the stomach was expanding (PLATES 15 and 16).

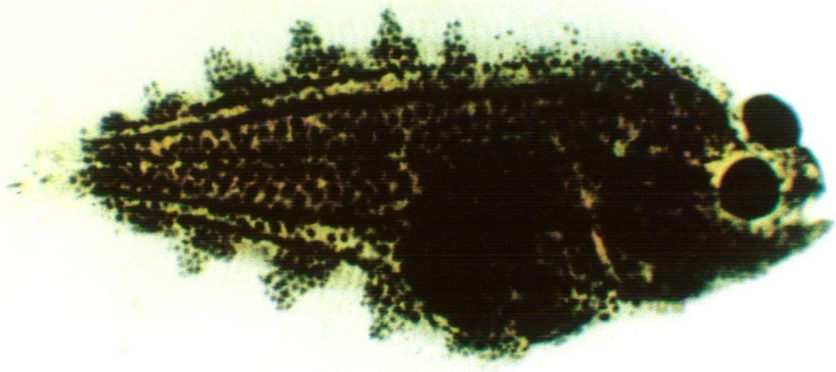

PLATE 15. External view of *R. tapirina* larva at day 16 post-hatch. Note: particularly advanced specimen showing start of eye migration, dark colouration and completion of tail flexion. Scale bar represents 1 mm.

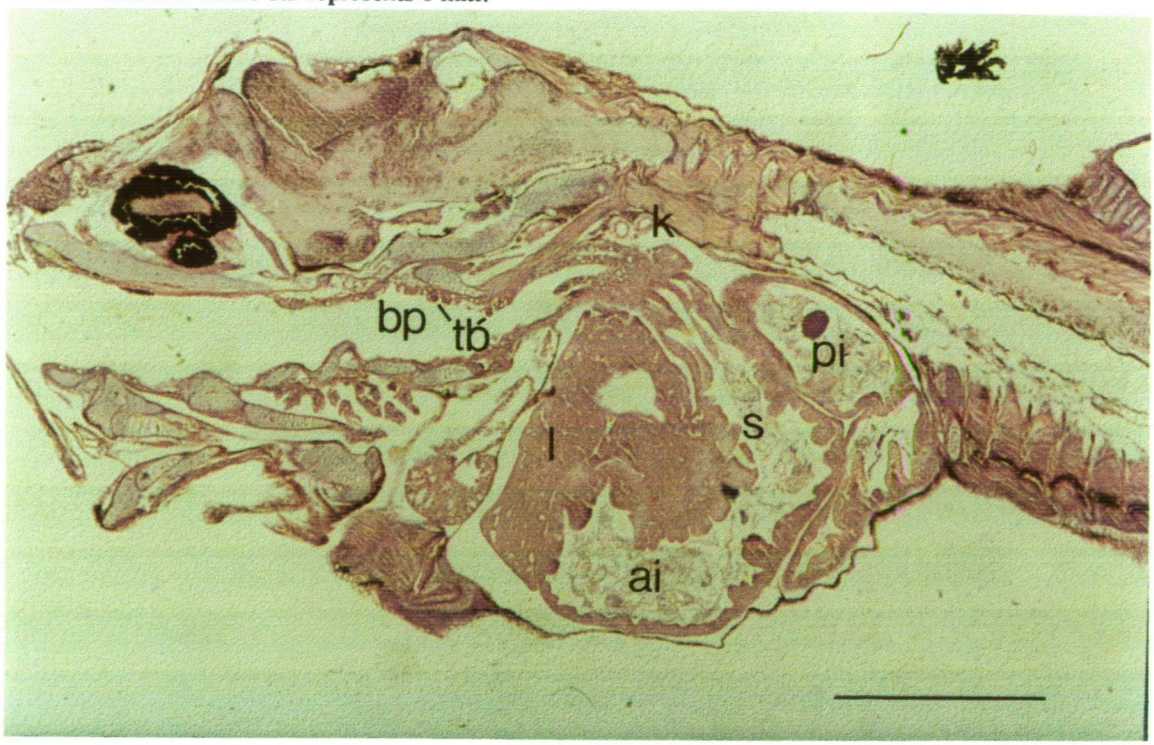


PLATE 16. Sagittal section of *R. tapirina* larva at day 16 post-hatch. k=kidney, ai=anterior intestine, pi=posterior intestine, bp=bucco-pharyngeal cavity, s=stomach, l=liver, tb=taste buds. Scale bar represents 500 µm.

By day 19-20 post-hatch the larvae were almost fully metamorphosed and lighter in colouration. Taste buds, teeth and stomach were fully formed (PLATES 17, 18 and 19). By day 30 post-hatch all larvae were fully metamorphosed (PLATE 20).

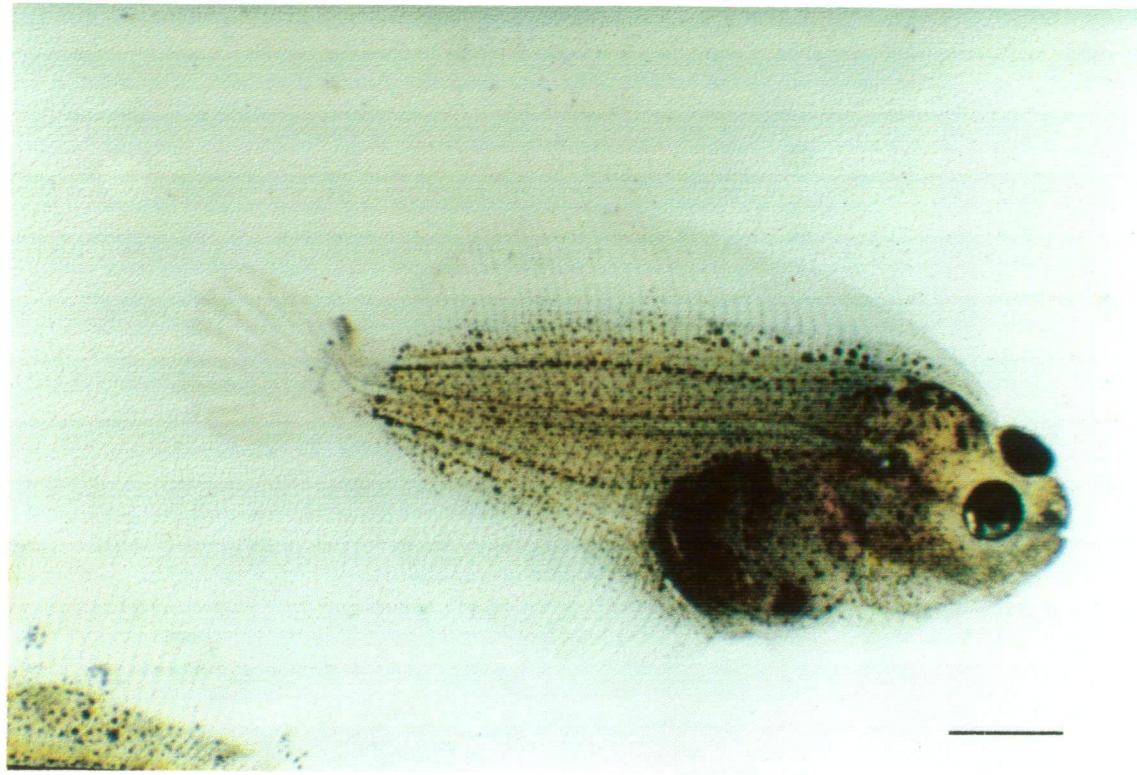

PLATE 17. External view of *R. tapirina* larva at day 19 post-hatch. *Artemia* are discernible in the gut. Scale bar represents 1 mm.

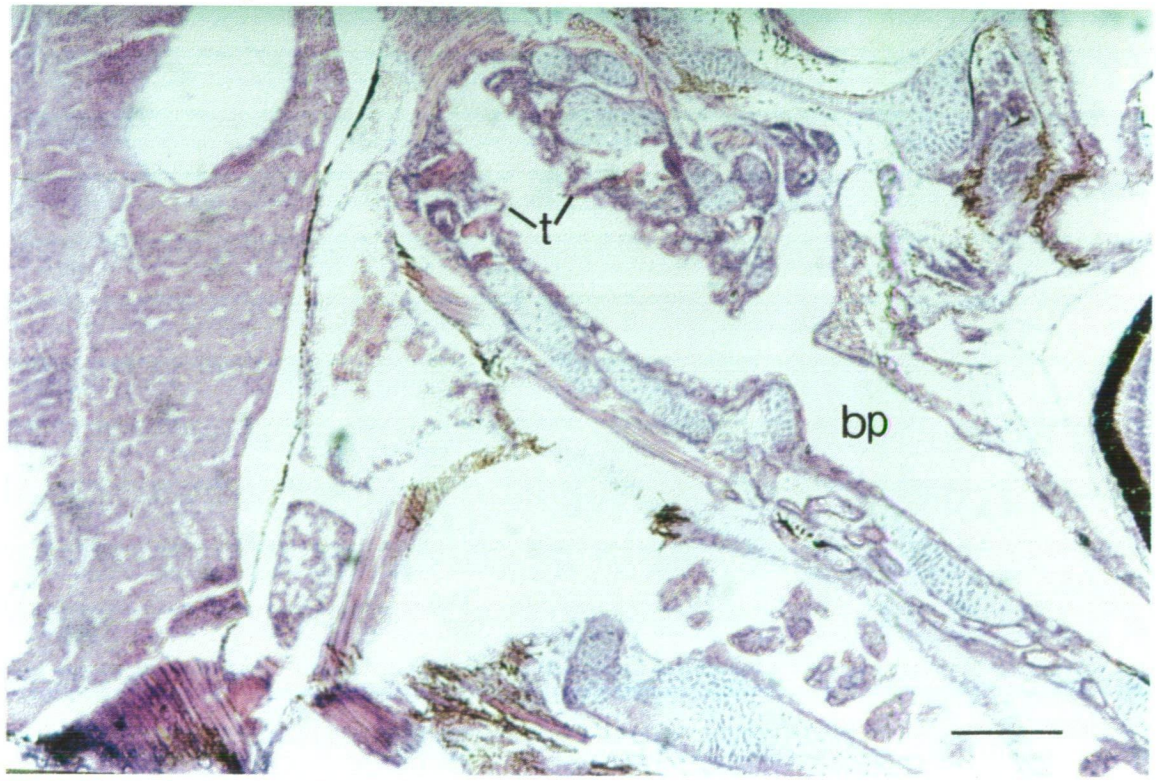


PLATE 18. Sagittal section of bucco-pharyngeal cavity of *R. tapirina* larva at day 19 post-hatch. t=teeth, bp=bucco-pharyngeal cavity. Scale bar represents 100 µm.


PLATE 19. Sagittal section of *R. tapirina* larva at day 20 post-hatch showing the enlargement of the stomach (s). e=eye, bp=bucco-pharyngeal cavity, ai=anterior intestine, pi=posterior intestine, pv=pyloric valve, l=liver, o=oesophagus. Scale bar represents 500 µm.

PLATE 20. External view of a fully metamorphosed *R. tapirina* juvenile at day 30 post-hatch. Scale bar represents 1 mm.

### 5.3.3. Gape size and feeding regime

The growth rate of *R. tapirina* larvae was constant over the 31 day period up to metamorphosis and the gape size increased proportionally with length (Fig 5.3.1.). The initial gape size was 429 ± 0 μm at day 4 post-hatch when fish length was 2.98 ± 0.04 mm ($\bar{x}$ ± s.e., n=10 larvae). Both the mouth and anus were open and the eyes were fully pigmented. Rotifers were observed in the guts of 90% of the sample at day 6 post-hatch, but although feeding behaviour was observed, rotifers could not be detected visually in the gut on either day 4 or 5 post-hatch. Instar II *Artemia* were ingested from day 10 post-hatch, when the gape size reached a height of 690 ± 0.02 μm and fish length was 4.71 ± 0.04 mm ($\bar{x}$ ± s.e., n=10 larvae). On day 12 post-hatch, 80% of the larvae contained *Artemia* in the gut and 100% in all samples thereafter. Gape size at day 20 post-hatch was 1,216 ± 0.03 μm and fish length was 8.51 ± 0.03 mm ($\bar{x}$ ± s.e., n=10 larvae). At day 28 post-hatch the gape height was 2,136 ± 0.07 μm and fish length was 10.89 ± 1.14 mm ($\bar{x}$ ± s.e., n=10 larvae).

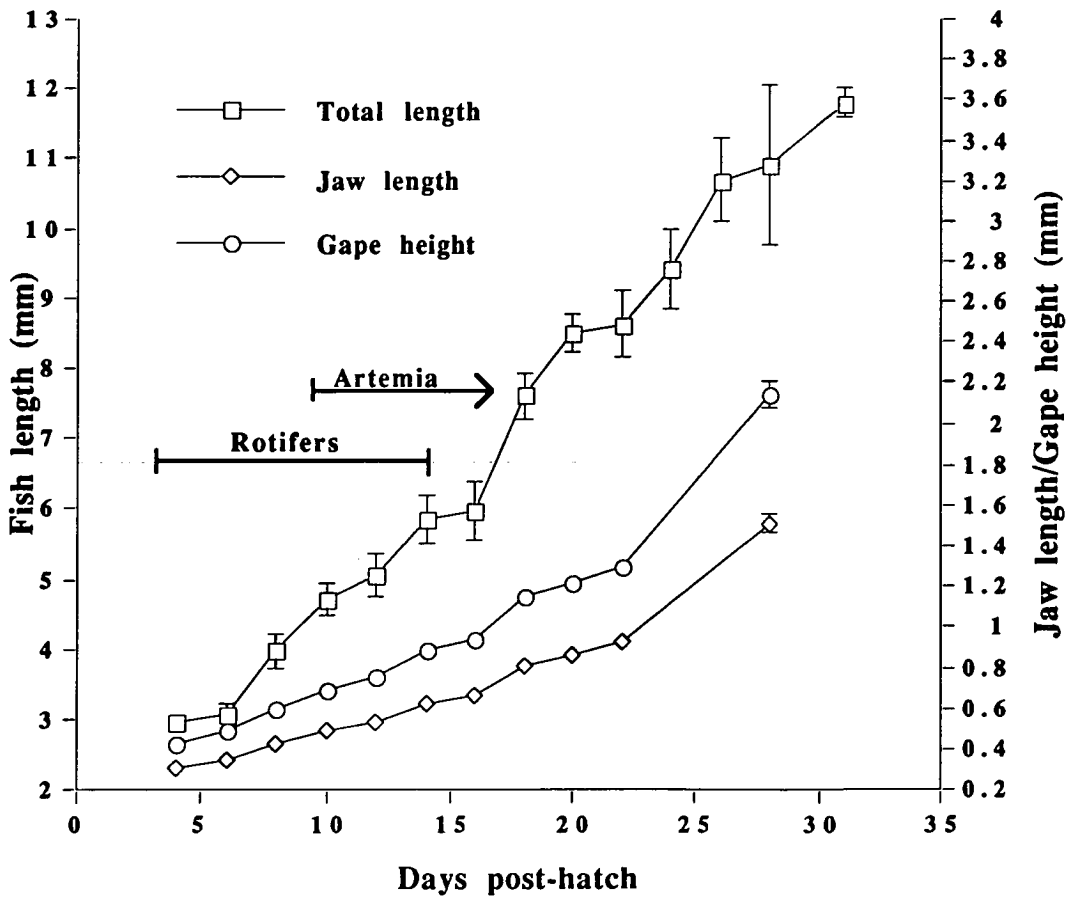


Fig. 5.3.1. Growth in length ($\bar{x}$ ± s.e., n=10 larvae), and increase in gape size ($\bar{x}$ ± s.e., n=10 larvae) of *Rhombosolea tapirina* larvae cultured at 15.5-16°C.

Points with no visible error bars have very small s.e.

### 5.3.4. The effect of different enrichment diets

#### (i) Experiment 1

The use of Frippak Booster as an enrichment diet for rotifers and *Artemia* resulted in significantly improved growth rates (Tables 5.3.2. and 5.3.3.). Significantly better growth in length and weight ($P<0.01$ and 0.05 respectively) was obtained using Frippak as an enrichment for live feeds. Growth in weight was significantly improved when compared with algal enrichment and 'Diet A' enrichment. There were no significant differences ($P<0.05$) in survival rates. No fish with malpigmentation of the ocular surface were observed in any of the treatments.

**Table 5.3.2. Initial and final weights and lengths, condition factors and survival rates for *R. tapirina* larvae fed live feeds enriched with different diets ($\bar{x} \pm$ s.e., n=3 replicates).**

| Enrichment diet. | Final length (mm) | Final weight (g) | Condition factor | Survival (%) |
|---|---|---|---|---|
| Frippak | 16.00 (0.15)$^a$ | 0.065 (0.008)$^a$ | 1.6 (0.2) | 63 (3) |
| Microalgae. | 14.28 (0.16)$^b$ | 0.040 (0.002)$^b$ | 1.4 (0.03) | 80 (7) |
| 'Diet A' | 14.58 (0.19)$^b$ | 0.036 (0.012)$^b$ | 1.1 (0.4) | 74 (6) |

Figures in the same column sharing a common superscript are not significantly different ($P>0.05$).

**Table 5.3.3. Results of one-way ANOVA comparing the final length, weight and survival (arc sine $\sqrt{}$ transformed) of *R. tapirina* fed with live feeds enriched with different diets.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 0.051 | 0.025 | 29.993 | **P < 0.05** |
| Error | 6 | 0.005 | 0.001 | | |
| Total | 8 | 0.056 | | | |
| Final weight | | | | | |
| Model | 2 | 0.0004 | 0.00020 | 11.444 | **P < 0.05** |
| Error | 6 | 0.0001 | 0.00002 | | |
| Total | 8 | 0.0005 | | | |
| Survival | | | | | |
| Model | 2 | 0.065 | 0.033 | 2.345 | **P > 0.05** |
| Error | 6 | 0.084 | 0.014 | | |
| Total | 8 | 0.149 | | | |

### (ii) Experiment 2

The results for Experiments 2, 3 and 4 were analysed using one-way ANOVA. However, as the treatments were unreplicated, the exact reason for significant differences occurring between tanks can only be assumed to be due to the diets. In some cases differences in stocking densities, due to mortalities may have had a greater effect than differences in diet. Using ANOVA to analyse these data is not strictly correct as individual fish in a tank cannot be assumed to be independent of one another, an assumption of ANOVA. However, differences between tanks can still be approximated using this method.

The enrichment diet had no significant effect ($P>0.05$) on the growth rate of *R. tapirina* larvae (Tables 5.3.4., 5.3.5. and Fig 5.3.2.). An improvement in growth rate was observed at day 62 in fish fed Frippak enriched prey but it was not significant ($P>0.05$). A 15% occurrence of malpigmentation on the ocular side was observed in fish fed Frippak enriched prey but not in the other treatments. It may be of significance that fish removed from the algal-enriched tank at day 15 for Experiment 1, and then fed on Frippak enriched feeds, did not show any incidence of malpigmentation. The growth rates observed in this trial were considerably lower than in later trials, although the reason for this is unclear.

**Table 5.3.4. Initial and final lengths, of *R. tapirina* larvae fed live feeds enriched with different diets ($\bar{x} \pm$ s.e., n=40 larvae).**

| Enrichment diet. | Length at day 1 (mm) | Length at day 13 (mm) | Length at day 34 (mm) | Length at day 62 (mm) |
|---|---|---|---|---|
| Frippak | 2.43 (0.04) | 4.43 (0.05) | 6.49 (0.10) | 11.2 (0.4) |
| Microalgae. | 2.43 (0.04) | 4.23 (0.05) | 6.48 (0.09) | 10.1 (0.3) |
| 'Diet A' | 2.43 (0.04) | 4.36 (0.09) | 6.75 (0.16) | 10.5 (0.3) |

**Table 5.3.5. Results of one-way ANOVA comparing the length at day 62 of *R. tapirina* larvae fed with live feeds enriched with different diets.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Model | 2 | 0.220 | 0.110 | 2.403 | **P > 0.05** |
| Error | 117 | 5.360 | 0.046 | Prob > F | |
| Total | 119 | 5.580 | | 0.095 | |

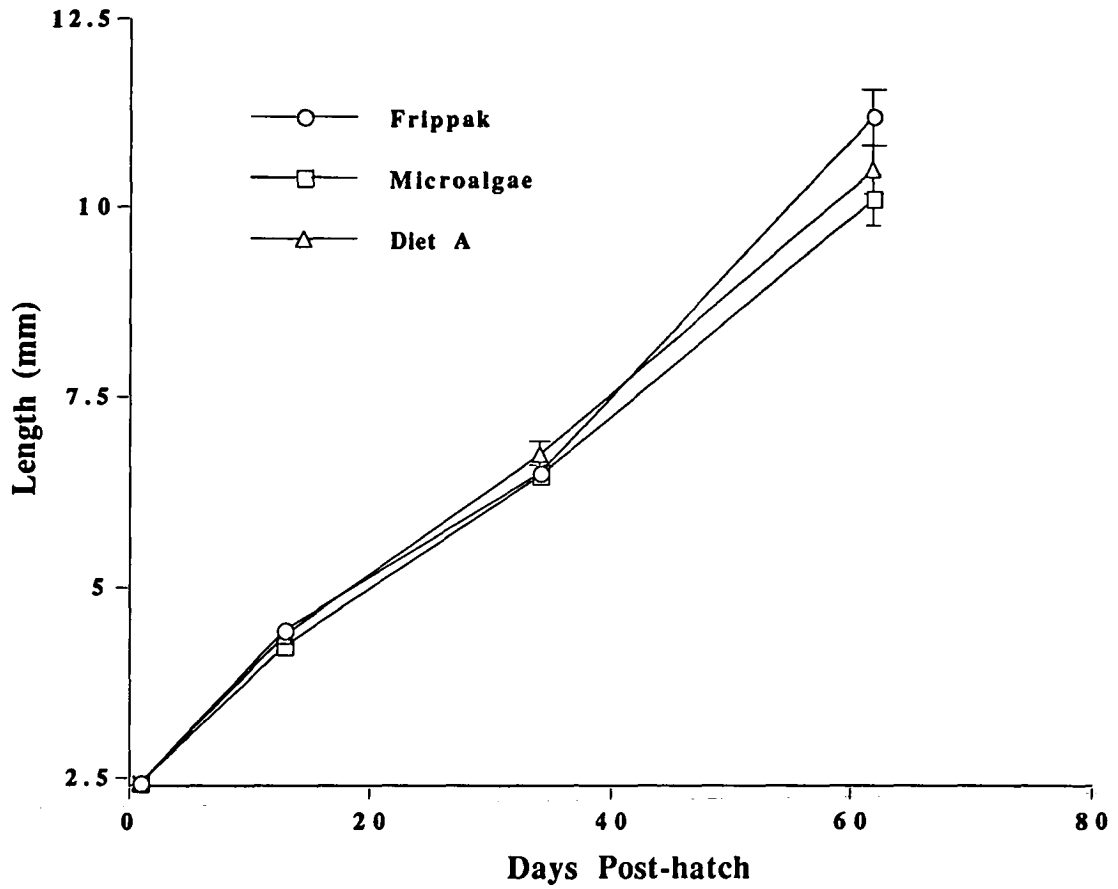


Fig. 5.3.2. Growth in length ($\bar{x} \pm$ s.e., n=40 larvae) of *Rhombosolea tapirina* larvae fed with live feeds enriched with different diets in 1991.

Points with no visible error bars have very small s.e.

### (iii) Experiment 3

The growth rates in this 1992 experiment were superior to any obtained previously and Frippak resulted in improved growth compared with algal enrichment (Tables 5.3.6., 5.3.7 and Fig 5.3.3.). The larvae fed Frippak enriched prey again showed significantly better ($P<0.05$) growth in weight, than those fed on algae enriched prey. Final lengths were not significantly different ($P>0.05$). Fish fed algae for the first 15 days showed a similar growth to those fed on Frippak enriched prey. The percentage of fish with white pigment on the ocular surface was similar in all three tanks.

**Table 5.3.6. Final lengths, weights and incidence of malpigmentation of *R. tapirina larvae* fed on live feeds enriched with different diets in 1992 ($\bar{x} \pm$ s.e., n=30 larvae).**

| Enrichment Diet | Final Length (mm) | Final Weight (g) | Malpigmentation (%) |
|---|---|---|---|
| Frippak | 12.4 (0.2) | 0.025 (0.001)$^{a}$ | 6 |
| Algae | 11.8 (0.2) | 0.021 (0.001)$^{b}$ | 7 |
| Algae + Frippak | 11.9 (0.2) | 0.022 (0.001)$^{ab}$ | 6 |

Figures in the same column sharing a common superscript are not significantly different ($P>0.05$).

**Table 5.3.7. Results of one-way ANOVA comparing the final length and weight of *R. tapirina* fed with live feeds enriched with different diets.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 7.42 | 3.708 | 2.701 | **P > 0.05** |
| Error | 87 | 119.43 | 1.373 | | |
| Total | 89 | 126.84 | | | |
| Final weight | | | | | |
| Model | 2 | 0.00034 | 0.00017 | 4.572 | **P < 0.05** |
| Error | 87 | 0.00320 | 0.00004 | | |
| Total | 89 | 0.00354 | | | |

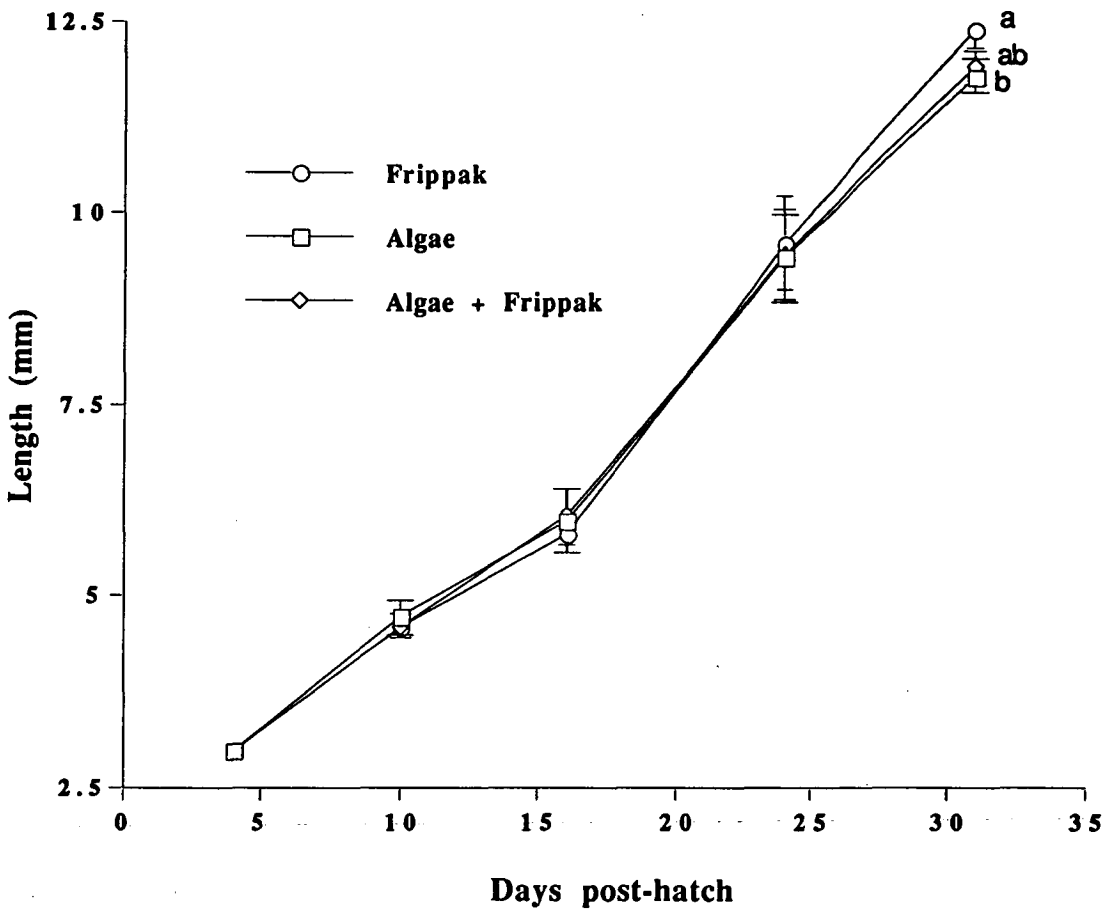


Fig. 5.3.3. Growth in length ($\bar{x} \pm$ s.e., n=30 larvae) of *Rhombosolea tapirina* larvae fed on live feeds enriched with different diets in 1992.

Points sharing a common superscript are not significantly different (P>0.05).

Points with no visible error bars have very small s.e.

### (iv) Experiment 4

The growth rates of *R. tapirina* larvae fed with live feeds enriched with different diets in 1993 are shown in Figure 5.3.4. The final weight of *R. tapirina* larvae fed Nutri-Pack enriched live feeds, was significantly higher ($P<0.05$) than larvae fed either, Frippak enriched or, algae enriched followed by Frippak-enriched live feeds. However, final lengths were not significantly different ($P>0.05$) (Tables 5.3.8 and 5.3.9). Growth rates in all tanks were lower than usual and survival in the tank fed algae enriched live feeds followed by Frippak enriched live feeds, appeared very low. High mortality occurred in this tank at around day 10 post-hatch. The reduction in growth rate after day 29 post-hatch may have been a sampling artefact on that day, as it is unlikely that the fish grew shorter. It may also have been due to tail-biting caused by lack of food (although this was not obvious in the sample) or selective mortality of larger fish.

The stocking density at day 31 appeared very high in the Frippak and Nutri-Pack enriched tanks and the quantity of *Artemia* required was underestimated resulting in poor growth. Malpigmentation rates in both the Frippak-enriched and the Nutri-Pack-enriched tanks were significantly higher ($P<0.05$) than in the tank fed algae enriched prey for the first 15 days. Due to zero variance in the Frippak data, the variances were heterogeneous and could not be transformed. However, the differences were highly significant and are therefore probably still valid. This is a different trend from that shown in Experiment 2.

**Table 5.3.8. Final length, weight and incidence of malpigmentation of *R. tapirina* larvae fed on live feeds enriched with different diets in 1993 ($\bar{x} \pm$ s.e., n=30 larvae).**

| Enrichment Diet | Final Length (mm) | Final Weight (g) | Malpigmentation (%) |
|---|---|---|---|
| Frippak | 6.92 (0.19) | 0.0052 (0.0004)$^a$ | 4 (0)$^a$ |
| Nutri-Pak | 7.24 (0.14) | 0.0065 (0.0003)$^b$ | 3 (1)$^a$ |
| Algae + Frippak | 7.25 (0.14) | 0.0052 (0.0004)$^a$ | 1 (0)$^b$ |

Figures in the same column sharing a common superscript are not significantly different ($P>0.05$).

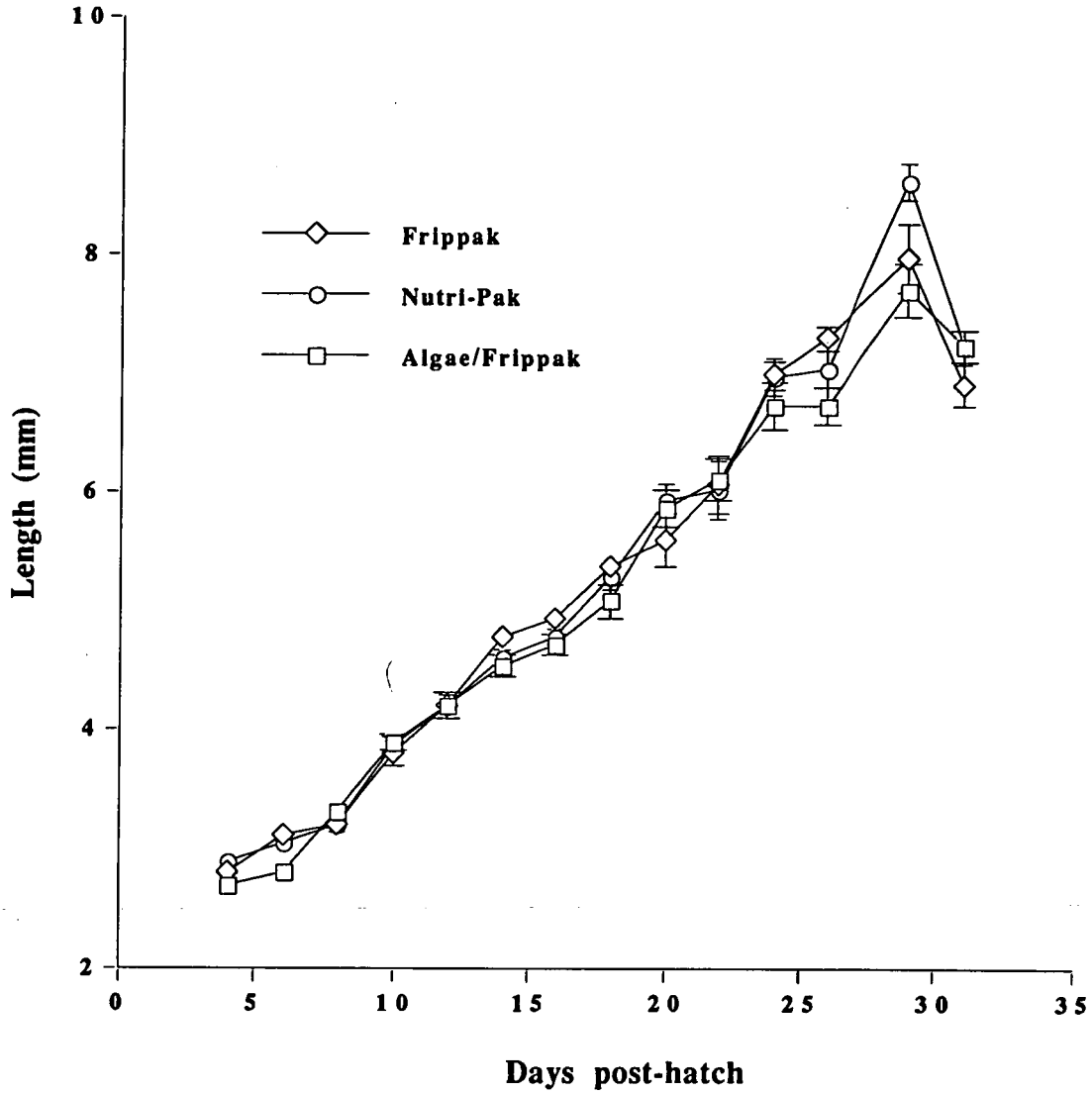


Fig. 5.3.4. Growth in length ($\bar{x} \pm$ s.e., n=30 larvae) of *Rhombosolea tapirina* larvae fed with live feeds enriched with different diets in 1993.

Points with no visible error bars have very small s.e.

**Table 5.3.9. Results of one-way ANOVA comparing the final length, final weight (arc sine √ transformed due to +ve skewness of data) and rate of malpigmentation (arc sine √ transformed data) of the ocular surface, of *R. tapirina* larvae cultured with live feeds enriched with different diets.**

| Source | DF | SS | MS | F Ratio | P value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 2.074 | 1.037 | 1.426 | **P > 0.05** |
| Error | 87 | 63.278 | 0.727 | | |
| Total | 89 | 65.352 | | | |
| Final weight | | | | | |
| Model | 2 | 0.00188 | 0.00094 | 5.419 | **P < 0.05** |
| Error | 87 | 0.01509 | 0.00017 | | |
| Total | 89 | 0.01697 | | | |
| Malpigmentation | | | | | |
| Model | 2 | 0.0212 | 0.011 | 17.89 | **P < 0.05** |
| Error | 6 | 0.0036 | 0.0006 | | |
| Total | 8 | 0.0247 | | | |

## 5.4. DISCUSSION

*Larval development*

In the greenback flounder (*Rhombosolea tapirina*) metamorphosis begins with a darkening of the skin at approximately day 15 and is complete by approximately day 30 at a length of 12.5 mm, in the fastest growing batches, at 15°C . In the study undertaken by Crawford (1984a), at a temperature of 12.7-16.5°C, 80% of the larvae of *R. tapirina* had settled on the bottom by day 63 post-hatch, and by day 85 post-hatch, all the larvae had completed metamorphosis at a mean length of 11.2 mm. At 16-17.4°C metamorphosis occurred after only 36 days, at a mean length of 10.7 mm. In the present study, metamorphosis occurred earlier and at a larger size, due probably to better feed composition, higher feed densities and higher water temperatures. Jones *et al.* (1974) cultured turbot (*Scophthalmus maximus*) at a temperature of 13.5-18°C, and observed metamorphosis between days 45 and 80 at a length of 23-30 mm, much larger than *R. tapirina*. Fonds (1979) investigated growth rates of *S. solea* at varying temperatures. At 22°C metamorphosis was completed on average, 14 days after first-feeding at a length of 9 mm. At 13°C metamorphosis occurred at 10 mm and 28 days after first-feeding.

Feeding behaviour in *R. tapirina* was observed as a 'shivering' motion and generally occurred at the water surface. After feeding had commenced, the larvae went through

a period up to day 15, during which they actively fed at the water surface and the looped gut developed further. Jones (1973) observed that the larvae of *S. maximus* assumed an S-shaped flexure of the body when feeding, from which they suddenly straightened and darted forward in a typical feeding motion. This behaviour was not observed in *R. tapirina*.

From day 15 onwards the pigmentation of *R. tapirina* larvae went through three distinct changes ending when metamorphosis was complete and the juveniles were feeding on the tank bottom, at approximately day 31. At day 15 post-hatch, the formation of large numbers of melanocytes in the dermis resulted in the larvae appearing black. This has also been recorded in *S. maximus* and is thought to be a protection from exposure to high light levels associated with the move to the surface (Grønås *et al.*, 1993). However, there was no obvious move to surface feeding with *R. tapirina* at this stage. In fact it was at this stage that the larvae began to spend more time lying on the tank bottom, coming up only occasionally to feed at the surface or in the water column.

At around day 20 post-hatch the larvae began to acquire numerous iridocytes producing a white spotted appearance against the black background. At this stage all the larvae were actively feeding on the tank bottom. This occurred at day 16 post-hatch in *S. maximus* (Grønås *et al.*, 1993). The final colour change involved a reduction in the iridocytes, possibly due to the increasing surface area of the larvae, and the production of red and yellow xanthocytes giving an overall brownish green juvenile colouration.

The development of the digestive tract in larval *R. tapirina* followed a very similar pattern to that of *S. maximus* reported by Cousin and Baudin-Laurencin (1985). At hatching the gut was a simple straight tube, which differentiated into posterior intestine and antero-median intestine, as a loop formed and feeding commenced. The antero-median intestine further differentiated into anterior intestine and median intestine, and finally the stomach increased in volume and probably became functional around day 20 post-hatch at 15°C. It has been suggested that the development of a functional stomach is associated secretion of pepsin and hydrochloric acid, and increases the digestive capacity of fish larvae, enabling them to digest artificial diets (Hjelmland *et al.*, 1993; Segner *et al.*, 1993). It may therefore, be possible to wean the larvae of *R. tapirina* onto a dry diet after day 20 post-hatch.

*Larval feeding*

Although *R. tapirina* are very small at first-feeding, approximately 3.0 mm, they have a large gape and are capable of ingesting rotifers from a population of normal size (80-240 µm). It was not shown whether they select the smaller individuals or ingest all sizes. Hunter (1984) suggested that first-feeding larval fish are capable of ingesting food particles up to 25% the width of the mouth. Assuming that mouth width is the same as gape height, when the mouth is fully open, then the larvae of *R. tapirina* should be able to ingest rotifers of 107 µm at first-feed as the gape is over 428 µm at this stage. The larvae of *S. maximus* show a preference for feed particles of less than 200 µm in length (Jones, 1972). Spectorova *et al.* (1974), showed that from a rotifer population of average size 227 µm, first-feeding larval *S. maximus maeoticus* picked out individuals of 126 µm average size. Larval length was about 3.5 mm at this stage (Spectorova and Doroshev, 1976), slightly larger than *R. tapirina* in the present study.

The change to instar II *Artemia* nauplii of 500-600 µm length occurred when the larval length reached 4.71 mm at day 9 post-hatch. With a gape of 690 µm at this stage the larvae should not be able to ingest particles of over 345 µm (50% of the gape height), according to Hunter (1984). However, Léger *et al.* (1986) suggest that the length of *Artemia* may not always be the criterion by which they are selected. The width of *Artemia* is approximately 300-400 µm and may be a more important measurement in terms of the ability of the larvae to ingest them.

Instar I *Artemia* nauplii were not used in the present study, but they could possibly be introduced to *R. tapirina* larvae at day 6-7 post-hatch, thereby reducing the reliance on rotifers. The changeover from rotifers to *Artemia* was successfully achieved by Crawford (1984a) with *R. tapirina*, by decreasing the concentration of rotifers between days 21 and 32, and increasing the numbers of *Artemia* nauplii. In the present study, Instar II nauplii were ingested by *R. tapirina* larvae at a very early age, compared to other species. Jones (1973) fed rotifers to *S. maximus* larvae until day 14 and introduced Instar I *Artemia* nauplii on days 12-14 post-hatch. Instar II *Artemia* were introduced on day 32. Person-Le Ruyet *et al.* (1981) fed rotifers to *S. maximus* from day 2 to day 13, instar I *Artemia* nauplii from day 7, and instar II *Artemia* nauplii from day 17.

There are several advantages in using instar II *Artemia* from an early age. They do not have to be good quality *Artemia*, as even cheap brands such as those from the Great Salt Lake can be used, providing they are enriched to improve their nutritional value. Watanabe *et al.* (1980) showed that freshwater *Artemia*, such as those from the Great Salt Lake, have a low content of n-3 highly unsaturated fatty acids (HUFA's) and are

therefore, unsuitable as a feed unless they are enriched. The harvesting of instar II *Artemia* is much simpler than that of Instar I nauplii, as it is not necessary to harvest before the yolk-sac is absorbed. Finally, the enzyme content of *Artemia* has been shown to increase after feeding and this may be of benefit to the fish larvae (Munilla-Moran *et al.*, 1990).

The nutritional quality of microalgae has been reviewed by Brown *et al.* (1989). The species of microalgae containing the highest levels of ecosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are *Isochrisis* (0.2 and 8.3%, respectively) and *Pavlova lutheri* (19.7 and 9.4%, respectively), which is why these species were used as enrichment diets in the present study.

It has been stated that early survival of marine fish larvae is enhanced by the addition of algae to the rearing tanks (Section 6.4.). This may be due to the algae either assimilating nitrogen metabolites or providing some essential nutrient (Jones, 1970). However, more recent work suggests that it is the quality of the zooplankton that is improved due to the ingestion of the microalgae and this improves their quality as a food organism. Howell (1979) showed that a mixture of six different microalgal species, when added to the larval tanks, did not sustain growth in *S. maximus* and after 5 days the larvae all died, whereas the control fish fed on rotifers, showed significant survival rates. He also demonstrated a significant improvement in larval growth rate and survival, when rotifers were added in the presence of the marine algae *Isochrisis galbana* rather than *Dunaliella tertiolecta*. Scott and Middleton (1979), showed that it is a deficiency in long-chain polyunsaturated fatty acids, rather than a toxin, that makes *D. tertiolecta* unsuitable as a food for rotifers. Larvae from tanks containing only *D. tertiolecta* contained lower levels of EPA and DHA than tanks containing other species of microalgae. The unhatched eggs of *S. maximus* however, contained higher levels of EPA and DHA than any other fatty acids. It seems probable that the larvae are unable to synthesise their own long-chain fatty acids and therefore rely on an external supply. It is not known whether the rotifers actually assimilate the long-chain fatty acids or just pass them on to the larvae as partly-digested algae.

In the present study an artificial diet containing freeze-dried *Spirulina* and developed for the culture of rotifers, by Gatesoupe and Luquet (1981) and Robin *et al.* (1984), was tested as an enrichment diet for 24 hour enrichment of both rotifers and *Artemia*. Results were acceptable, but not comparable to those achieved with Frippak. These results show that the nutritional quality of rotifers can be improved with this diet. The diet has also been shown to be a suitable diet for the mass culture of rotifers (Robin *et al.*, 1984; Daintith, pers. comm.). It therefore appears possible to completely eliminate fresh algae from a commercial culture system, thereby reducing the cost of production

as well as the initial cost of setting up the facility. The culture of rotifers is discussed in Fulks and Main (1991). A major constraint on production was identified as the cost of setting up facilities for, and producing, fresh algae as a feed. Consequently the development of artificial diets was considered a priority for future research.

In the present study, the enrichment of live feeds with an artificial enrichment diet improved larval growth as was expected. Algae and diet 'A' gave very similar results. The use of Frippak resulted in higher mortality and appeared to increase the rate of malpigmentation in some experiments, when used as an enrichment for rotifers in the first 15 days post-hatch, but it did result in improved growth. Watanabe *et al.* (1978, 1980) showed that the HUFA's, particularly EPA and DHA, are essential for the survival of marine fish larvae. Frippak was shown by Rimmer and Reed (1989) to increase the levels of HUFA, particularly EPA (7-9%) and DHA (1-2%), in *Artemia*. They also showed that it resulted in increased survival of barramundi (*Lates calcarifer*) larvae through metamorphosis. Frippak contains 9.29% EPA and 13.21% DHA according to the manufacturer's specifications, although Sweetman (1992) records levels of only 5.7 and 3.4% respectively, in rotifers enriched for 24 hours. The higher levels of DHA than in the microalgae used in this study may account for the improved performance of the larvae. Nutri-Pack is also a high HUFA enrichment product and resulted in similar results to those shown for Frippak. No specifications for this diet are available, but it is an extremely cheap form of enrichment.

In the present study the live feeds were fed to the fish twice daily with further enrichment between the first and second feed. There was some evidence to suggest that poorer growth resulted from feeding only once per day in Experiment 4 (section 5.3.4.). Benavente and Gatesoupe (1988) investigated the effect of starving rotifers, on the content of n-3 HUFA. Enriched rotifers containing 2% n-3 HUFA, on a dry weight basis, were fed to larvae of *S. maximus* either in one feed, which meant that they slowly starved during the day, or semi-continuously, over a 12 hour period, during which further enrichment took place. Although the survival and growth rates were not significantly different, the continuously fed larvae did contain a higher levels of total lipid and DHA, which may have had an effect on later development.

In the present study *Artemia* from the Great Salt Lake were used. These have been shown to have a poor HUFA content (Watanabe *et al.*, 1980), but they are much cheaper than high quality strains. When used after 24 hour starvation and enriched, they proved to be an effective feed for *R. tapirina*. There was some evidence to suggest that improved growth rates resulted from removal of rotifers and *Artemia* from the larval rearing tanks prior to the addition of each newly enriched batch. Ablett and Richards (1980) showed that if *Artemia* are unfed for 48 hours their suitability as a

food organism decreases considerably. It was thought that the hatching temperature of the *Artemia* affected their nutritive value, with 30°C and 24 hour hatching, being better than 23°C and a 48 hour hatching period. It has also been stated that *Artemia* become less visible after the yolk-sac is absorbed and this is the reason for the decrease in quality with age.

In the present study a feed rate of 10-20 rotifers/ml followed by *Artemia* at a density of 1/ml increasing to 3/ml by day 20, was successfully used to culture *R. tapirina* larvae with good growth and survival. The density of food organisms in the tanks is an important aspect of early larval feeding, as it affects larval growth and survival as well as the cost of production. Crawford (1984a) found that a density of 1.6 rotifers/ml up to day 20, followed by 0.5-1 *Artemia* nauplii/ml until weaning after metamorphosis, gave good survival and growth in *R. tapirina*. In the long-snouted flounder (*Ammotretis rostratus*) however, a density of 0.8 rotifers/ml from days 5-7 from hatching, resulted in almost total mortality, probably due to starvation. A concentration of 10 rotifers/ml has been used successfully for the rearing of larval *S. maximus* (Howell, 1979) and the same concentration of *Artemia* was successfully used for the rearing of first-feeding larval *S. solea*. (Gatesoupe and Luquet, 1981/1982). Spectorova *et al.* (1974) experimented with different concentrations of rotifers as food for *S. maximus maeoticus*. Using concentrations of 0.1, 0.5, 1, 5 and 10 rotifers/ml there was very little difference in the percentage survival between groups, but at a level of 1 rotifer/ml, the larvae contained 2.9 rotifers in the gut after 2 hours, which was slightly more than at other concentrations. It was also shown that at 16-17°C complete digestion occurs in 3-5 hours. Saksena and Houde (1972) found that concentrations of 1-2 copepods/ml were sufficient for the rearing of larval scaled sardine (*Harengus pensacolae*) and bay anchovy (*Anchoa mitchilli*). They concluded that a higher concentration of food organisms is required in the laboratory than in the wild, due to behavioural changes in the larvae. The high larval density in laboratory studies must also increase the required feed density. Houde (1977) found that 1 copepod/ml or higher was sufficient for rearing larvae of lined sole (*Achirus lineatus*).

In the present study a feeding rate of 1-5 *Artemia*/ml was used for feeding *R. tapirina* after the rotifer stage, but at high stocking densities this was found to be inadequate. Under these conditions it would have been better to continuously monitor the feed density, either by sampling or visually, followed by the addition of *Artemia* as necessary. Crawford (1984), used a concentration of 0.5-1 *Artemia* nauplii/ml for rearing larval *R. tapirina*. Werner and Blaxter (1981) conducted experiments into the effects of prey density on the growth of larval herring (*Clupea harengus*) and found the yield, in terms of mean dry weight of survivors multiplied by the number of survivors, was highest at densities of 0.3-1 *Artemia* nauplius/ml and declined when the

density was raised to 3.0 *Artemia*/ml. A concentration of 10 nauplii/ml was used for larval *S. solea* (Gatesoupe and Luquet, 1981/1982) and Japanese flounder (*Paralichthys olivaceus*) (Kuronuma and Fukusho, 1984). In an experiment with *D. labrax*, at a stocking density of 50 larvae/l, Barahona-Fernandes and Conan (1981) assessed the daily intake of *Artemia* between days 10 and 75. They found that feed intake was approximately 40-64% of their own weight per day on a dry weight basis.

*Pigmentation*

The levels of pigmentation on the ocular surface of *R. tapirina* were considerably affected by the enrichment diets used with the live feeds. The problem of malpigmentation is common to all cultured flatfish species and is a serious threat to their marketability (Grønas *et al.*, 1993). Pigmentation or melanin production is under the control of the hypothalamus which acts on the pituitary in the presence of light. The pituitary secretes melanin-stimulating hormone (MSH) (Grønås *et al.*, 1993). Seikai *et al.* (1987a) and Fukusho *et al.* (1987) found that *P. olivaceus* larvae were most sensitive to malpigmentation problems in the early larval stage (stage D) the beginning of the caudal fin ray formation, this is similar to the result obtained in the present experiment as enriching with algae during the first 15 days of larval rearing, appeared to reduce the level of malpigmentation. Seikai *et al.* (1987b) observed different levels of malpigmentation in *P. olivaceus* fed with different strains of *Artemia*. However, they could not identify the cause. Kanazawa (1991) showed that DHA, vitamin A palmitate and phospholipid were essential components in the diet reducing the level of malpigmentation, but only if more than one of these compounds was limiting at the same time. Their conclusion was that nutritional deficiencies caused malformation of the rhodopsin in the rod cells of the eye, which prevented visual cues from being detected and interrupted the secretion of MSH. Kanazawa (1993) showed that abnormally pigmented *P. olivaceus* had weak visual perception and could not differentiate between dark and light sections of an experimental tank. If microparticulate diets or rotifers enriched with vitamin A, DHA and phospholipid are fed to flatfish, 10 days after hatching, then albinism can be prevented.

Izquierdo *et al.* (1992) observed an increasing level of malpigmentation in larval *P. olivaceus* with increased HUFA levels. Grønås *et al.* (1993) mention that melanin functions to neutralise reactive chemical moieties generated by both photic exposure and oxidative metabolism. Excessive HUFA levels may result in an increase in these by-products which may have an effect on dermal pigmentation, perhaps by causing melanin to be transferred from the dermis to the internal organs for protection. It may have been this effect that was shown in the present study when using high HUFA enrichment products such as Frippak and Nutri-Pack.

A further function of melanin in fish is to protect macrophages associated with the immune system (Grønås *et al.*, 1993). If this is the case then a similar transfer of melanin from the dermis to the macrophages may occur during disease outbreaks or in the presence of the high bacterial levels associated with the use of high HUFA diets. The position of the dark patches on the blind side of the juveniles could support this conclusion. The main areas of dark pigmentation occur around the edge of the body, around the gut and on the fins and lower opercula (PLATE 21, p. 164). These are all areas of abrasion and potential sites of infection by pathogens such as *Flexibacter maritimus*, a pathogen which has been detected on larvae. Melanin could be transferred to these areas to facilitate resistance to disease. Seikai and Matsumoto (1991) suggest that there are at least three factors controlling the differentiation of pigmentation to the ocular and blind sides: 1) a built in clock which causes differentiation to occur at a certain stage during development; 2) tissue-detected environmental cues which appear to be different on either side of the body; and 3) external factors. As light is the main cue for dermal melanin production (Grønås *et al.*, 1993; Kanazawa, 1993), this may be important, particularly as light levels are obviously different on the ocular side from the blind side. It seems possible that the lack of pigment on the ocular side of some *R. tapirina* juveniles is caused by nutritional deficiencies or incorrect light levels, while the dark pigment on the blind side is the result of abrasion and a resultant increase in melanin.

The results of the present study indicate that:

1. The larvae of *R. tapirina* require marine rotifers for first-feeding but Instar II *Artemia* nauplii can be ingested after day 9 post-hatch.

2. Metamorphosis of *R. tapirina* larvae occurs at approximately day 30 post-hatch at 15°C and the larvae undergo a series of colour changes as successive pigment types are formed.

3. The digestive tract of *R. tapirina* develops in the same manner as that of other flatfish larvae, the stomach becoming functional at around day 20 post-hatch.

4. Enrichment of live feeds with high HUFA diets results in improved growth rates of *R. tapirina* larvae, but pigmentation can be improved by the use of microalgae during the first 15 days post-hatch.

5. A juvenile size of around 12 mm length and 0.025 g weight can be achieved at metamorphosis with survival rates of approximately 15% from hatching.

# CHAPTER 6

# WEANING TO AN ARTIFICIAL DIET

## 6.1. INTRODUCTION

The use of live feeds in marine fish larviculture is both universal and essential. Live feeds stimulate first-feeding and promote good growth and survival of larvae. However, the production and use of live feeds is expensive, complicated, and has a number of undesirable side effects: 1) the bacteria associated with zooplankton cultures can be detrimental to fish larvae; 2) the nutritional value of the live feeds is extremely variable; 3) the waste products from the zooplankton can increase the load on the fish rearing system; and 4) the outlet screens have to be of very small pore size to avoid losing expensive zooplankton and therefore, flow rates have to be low to avoid blocking by the zooplankton resulting in overflows.

For these reasons it is considered highly desirable to be able to use artificial diets for the feeding of fish larvae. At present it is only the salmonids that are reared from first-feeding exclusively on dry artificial diets (Jones *et al.*, 1991). There have been very few successful attempts to feed the larvae of marine fish on artificial diets from first-feeding and these have always resulted in low survival and poor growth (Adron *et al.*, 1974; Applebaum, 1985). It appears that the main problems with artificial diets are their unacceptability to the larvae and low digestibility. Associated technical problems include: the lack of buoyancy of the diets, and their instability in water (Jones *et al.*, 1991).

The short digestive tracts of carnivorous larval fish allow only very short retention times and therefore the diet must be highly digestible (Jones *et al.*, 1991). It has been shown by a number of authors that the digestive enzyme system of early larvae is very primitive and the digestion of food is assisted by autolysis of the prey and enhanced by the enzymes contained within the prey (Tanaka *et al.*, 1972; Lauff and Hofer, 1984; Clark *et al.*, 1986; Clark *et al.*, 1987; Uys *et al.*, 1987; Hjelmeland *et al.*, 1988; Munilla-Moran and Stark, 1989; Munilla-Moran *et al.*, 1990). The formation of the stomach and the commencement of peptic enzyme production appears to be critical to successful digestion of conventional artificial diets (Segner *et al.*, 1993).

It has been found that dry or microencapsulated diets are ingested by larvae at a lower rate than live feeds (Tandler and Kolkovski, 1991; Weinhart and Rösch, 1991). Walford *et al.* (1991) showed that the protein coat of microcapsules was not digested even though the particles where readily ingested by barramundi (*Lates calcarifer*) from first-feeding. However, if they were fed in conjunction with rotifers the protein coat was absorbed. Sorgeloos and Léger (1992) are of the opinion that there are so many technological difficulties associated with designing artificial diets for marine fish larvae, that they may never be a cost effective live feed replacement.

The attractiveness and palatability of the weaning diet are critical factors in their formulation. Extracts from natural products such as squid can increase the attractiveness of diets (Mackie and Mitchell, 1985). Person-Le Ruyet *et al.* (1983), experimented with inosine in an extruded moist pellet as an attractant for larval turbot (*Scophthalmus maximus*) after a period of feeding on live feeds. Specific growth rate was increased from 10-13% per day if the diet was fed for at least 10 days. A mixture of betaine, glycine and inosine was used successfully by Metaillier *et al.* (1983b) as a chemical attractant for weaning the larvae of sole (*Solea solea*) after initial feeding on live feeds.

It has been shown that the condition of the larvae prior to weaning is the most important factor in determining the success of the weaning process (Bromley and Howell, 1983; Person-Le Ruyet *et al.*, 1993). Condition was measured in terms of growth rates and therefore, the nutritional quality of the live feeds offered to the early larvae is of paramount importance. The enrichment used for live feeds has an obvious effect on their nutritional quality, and subsequent weaning success of the larvae.

Crawford (1984a) weaned the newly metamorphosed larvae of the greenback flounder (*Rhombosolea tapirina*) onto 0.5 mm trout crumbles. Three methods were compared; 1) a direct transfer from live to artificial feeds; 2) frozen or freeze-dried *Artemia* for the first 10 days of weaning, followed by trout crumbles and freeze-dried *Artemia* for the next 10 days and finally trout crumbles on their own; and 3) slowly decreasing the live *Artemia* and feeding increased numbers of freeze-dried *Artemia*, mixed with trout crumbles, over a 4 week period. Complete mortality was observed when a direct transfer was used and 30% mortality occurred 7-10 days after weaning, when using the other two methods.

The specific objectives of this study were:

1. To assess the effects of available weaning diets on growth and survival of *R. tapirina*, in order to ensure that a suitable diet was used in future weaning experiments.

2. To assess the effects of different stocking densities on growth and survival of *R. tapirina* during weaning, in order to ensure that a suitable stocking density was used in future weaning experiments.

3. To investigate the effects of different periods of overlap between live and artificial diets, on growth and survival of *R. tapirina* during weaning, in order to determine the optimum changeover period.

4. To investigate the effects of fish age and size on growth and survival of *R. tapirina* during weaning, in order to determine the optimum age and size to commence weaning.

5. To examine the effects of different live feed enrichment diets for larval *R. tapirina* on subsequent growth and survival during weaning, in order to determine the optimum enrichment to use for the larvae prior to the commencement of weaning.

6. To examine the effect of partial starvation of *R. tapirina* larvae on subsequent growth and survival during weaning, in order to determine whether this is an effective strategy for optimisation of weaning success.

## 6.2. MATERIALS AND METHODS

All the experiments in this Chapter were carried out in a set of nine black hemispherical fibreglass tanks of 25 l capacity, in a recirculation system incorporating filtration equipment (Appendix 1.3.). Unless otherwise stated, a 24 hour light regime was provided by overhead fluorescent tubes, giving a light intensity of 650-750 lux at the water surface. Temperature was maintained between 15 and 17°C. Flow rates were set at 30 l/hour. In experiments prior to 1992, live feeds were enriched with the microalgae *Isochrisis* sp. (Tahitian clone) and *Pavlova lutheri*, up to and during weaning, unless otherwise stated (Appendix 1.2.). After 1993 Frippak Booster was used as the main enrichment. The weaning phase was initiated using the schedule outlined by Devresse *et al.* (1991) for use with Lansy weaning diets (Artemia Systems SA, Gent, Belgium; Table 6.2.1.).

**Table 6.2.1. Feeding regime used in weaning experiments. Size 1 was the smaller particle size and size 2 the larger (Table 6.2.2.) (from Devresse *et al.*, 1991).**

| Days from start of weaning | *Artemia* $10^6/m^3/d$ | $g/m^3/d$ | Amount of dry food fed Proportion (%) | |
|---|---|---|---|---|
| | | | Size 1 | Size 2 |
| 1-3 | 20 | 20 | 100 | |
| 4-7 | 20 | 30 | 100 | |
| 8-11 | 15 | 40 | 100 | |
| 12-15 | 10 | 60 | 70 | 30 |
| 16-20 | 5 | 80 | 50 | 50 |
| 20-24 | 0 | 100 | 30 | 70 |
| 25-30 | 0 | 120 | | 100 |
| 31-39 | 0 | 180 | | 100 |
| 39 on | 0 | 10% of biomass | | 100 |

This schedule is for a 20 day weaning period, but was reduced to 10 days for most experiments and 5 days in the experiment described in section 6.2.4. The length of the weaning period is stated in the individual sections. The artificial feed used in all experiments (except that described in section 5.2.2.) was a 1:1 mixture of Biodiet No. 1 (Bioproducts Inc., Oregon, USA; particle size = 300-500 μm, proximate composition = 43% protein, 14.5% fat) and Skretting 0.6G (T. Skretting A/S, Stavanger, Norway; particle size = 600 μm, proximate composition = 51.7% protein, 17.3% fat).

Feeding with artificial feed was carried out three times daily, while *Artemia* was fed once only, just after the third feed of artificial food. All experiments were carried out with three, randomly selected, replicate tanks assigned to each treatment. The base of each tank was siphoned daily to remove dead fish, uneaten food and faeces. The dead fish were counted in order to assess the daily pattern of mortality during weaning. There was normally a small discrepancy between the number of fish counted in and the number recovered, either dead or alive, and so the survival depicted in the graphs is observed survival and varied slightly from actual survival, which is given in the tables and was calculated by the formula:

$$\text{actual survival (\%)} = \frac{\text{initial number of fish counted in}}{\text{live fish remaining at the end of the experiment}} \times 100$$

An initial length and weight was calculated for each batch of fish used in the experiments, by individually weighing and measuring a sample of 30-50 fish. Subsequent samples of 20 fish from each tank were weighed and measured at intervals during the experiments and a final sample of 30 fish was weighed and measured for use in the statistical analyses as outlined in Appendix 1.4.

Experiments are numbered in this section to avoid confusion due to the number of sub-headings.

### 6.2.1. Experiment 1. - Weaning diets

This experiment was designed to evaluate the performance of three different diets as weaning diets for *R. tapirina*. Each tank was stocked with 125 (5/l) juvenile flounder of 82 days post-hatch (initial length = 16.4 ± 0.4 mm, weight = 0.067 ± 0.004 g; $\bar{x}$ ± s.e., n=30 fish). A 12:12 light: dark cycle was provided in this experiment due to another experiment requiring this cycle, being conducted in the same room. All fish were allowed to acclimatise for 6 days while being fed on microalgae enriched *Artemia*, at a rate of 5 animals/ml. After this period the 20 day weaning phase was

initiated. The tanks were arranged randomly with three replicate tanks assigned to each of the experimental diets. Details of the diets used are given in Table 6.2.2.

**Table 6.2.2. Particle sizes and proximate composition of diets used in Experiment 1 (Section 6.2.2.).**

| Diet | Product name | Particle size small (μm) | Particle size large (μm) | Protein content (%) | Fat content (%) |
|---|---|---|---|---|---|
| 1 | Lansy A2 and W3 | 150-300 | 300-500 | 50 | 15.5 |
| 2 | Sevbar Nos. 1 and 2* | 200-300 | 300-500 | 50 | 8 |
| 2 | Biodiet No. 1 and Skretting 0.6G | 300-500 | 600 | 43<br>51.7 | 14.5<br>17.3 |

* Sanofi Aquaculture, Paris, France.

The experiment was terminated at day 133 post-hatch (45 days from the start of the experiment), when the daily mortality rate had fallen to a steady low level. Measurements of the weight and length of 20 fish from each tank were recorded at regular intervals during the experiment. Mortalities were recorded daily, and at the termination of the experiment, the survivors were counted and a random sample of 30 fish from each tank was individually weighed and measured.

### 6.2.2. Experiment 2. - Stocking density

An experiment was undertaken to investigate whether the low stocking density used (5 fish/l) in Experiment 1 (Section 6.2.2.) was responsible for the low survival rates obtained. Each of three replicate tanks were stocked with either 125 (5/l), 250 (10/l) or 500 (20/l) juvenile flounder of 47 days post-hatch (initial length = 13.9 ± 0.3 mm, weight = 0.034 ± 0.002 g; $\bar{x}$ ± s.e., n=30 fish) previously fed with rotifers and *Artemia* enriched with microalgae (Appendix 1.2.). The artificial diet used was a 1:1 mix of Biodiet No. 1 and Skretting 0.6G with a 20 day weaning period. The experiment was terminated at day 97 post-hatch (50 days from the start of the experiment). Measurements of the weight and length of 20 fish from each tank were recorded at regular intervals during the experiment. Mortalities were recorded daily, and at the termination of the experiment, the survivors were counted and a random sample of 30 fish from each tank was individually weighed and measured.

### 6.2.3. Experiment 3. - Length of the changeover period

An experiment was undertaken in 1993 to investigate the effect of different weaning periods on subsequent growth and survival. Each tank was stocked with 200 fish of 28

days post-hatch (initial length = 7.86 ± 0.2 mm, weight = 0.0073 ± 0.0005 g; $\bar{x}$ ± s.e., n=50 fish) previously fed on rotifers and *Artemia* enriched with Frippak Booster. Weaning periods of 5, 10 and 20 days were randomly assigned to each of the nine tanks giving three replicates for each treatment. All fish were the same age at the initiation of weaning. The 5 day and 10 day weaning schedules are shown in Tables 6.2.3. and 6.2.4.

**Table 6.2.3. Weaning schedule for 5 day weaning of *R. tapirina* (adapted from Devresse *et al.*, 1991).**

| Days from start of weaning | *Artemia* (No/ml/d) | Artificial food ($g/m^3/d$) |
|---|---|---|
| 1 | 10 | 24 |
| 2 | 5 | 24 |
| 3 | 5 | 24 |
| 4 | 5 | 40 |
| 5 | 2.5 | 72 |
| 6-18 | 0 | 80 |
| 19-32 | 0 | 100 |
| 33+ | 0 | 10‰ biomass |

**Table 6.2.4. Weaning schedule for 10 day weaning of *R. tapirina* (adapted from Devresse *et al.*, 1991).**

| Days from start of weaning | *Artemia* (No/ml/d) | Artificial food ($g/m^3/d$) |
|---|---|---|
| 1-2 | 10 | 24 |
| 3-4 | 5 | 24 |
| 5-6 | 5 | 24 |
| 7-8 | 5 | 40 |
| 9-10 | 2.5 | 72 |
| 11-18 | 0 | 80 |
| 19-32 | 0 | 100 |
| 33+ | 0 | 10‰ biomass |

The artificial diet used was a 1:1 mix of Biodiet No. 1 and Skretting 0.6G. The experiment was terminated at day 76 post-hatch (48 days from the start of the experiment). Measurements of the weight and length of 20 fish from each tank were recorded at regular intervals during the experiment. Mortalities were recorded daily and at the termination of the experiment, the survivors were counted and a random sample of 30 fish from each tank was individually weighed and measured.

### 6.2.4. Experiment 4. - Age and size at weaning

#### (i) Weaning at days 43, 51 and 59 post-hatch.

This experiment was initiated in order to investigate whether weaning earlier and at a smaller size, affected weaning success. Each tank was stocked with 225 metamorphosed flounder of 33 days post-hatch (length = 16.5 ± 0.3 mm, weight = 0.063 ± 0.003 g; $\bar{x}$ ± s.e., n=50 fish). These larvae were a mixed batch of fish reared with different enrichment diets as described in section 5.4.6. (iii) and randomly assigned to the 25 l tanks. They were also the same batch as used in experiment 5.2.6. (i). All fish were fed at a rate of 10 Artemia /ml/d until weaning began and thereafter a 10 day weaning schedule was used as described in Table 6.2.4. (A feed rate of 10 *Artemia* /ml/d equates to approximately 1,000 *Artemia* /fish/d).

Weaning commenced on day 43 post-hatch for three randomly selected tanks. Weaning of the second set of three tanks commenced on day 51 and the third set was weaned from day 59 post-hatch. The artificial diet used was a 1:1 mix of Biodiet No. 1 and Skretting 0.6G. The experiment was terminated at day 89 post-hatch (58 days from the start of the experiment). Measurements of the weight and length of 20 fish from each tank were recorded at regular intervals during the experiment. Mortalities were recorded daily, and at the termination of the experiment, the survivors were counted and a random sample of 30 fish from each tank was individually weighed and measured.

#### (ii) Weaning before metamorphosis

To further investigate the effect of early weaning an experiment was undertaken to discover whether weaning could be initiated before metamorphosis was completed and what effect this would have on growth and survival. Three batches of 107 larvae at 23 days post-hatch (initial length = 8.71 ± 0.21 mm, initial weight = 0.016 ± 0.001 g; $\bar{x}$ ± s.e., n=30 fish) were placed in the 25 l system. These larvae were at stage 5 M (Appendix 1.5). Weaning was carried out using the 10 day schedule (Table 6.2.4.). The artificial diet used was a 1:1 mix of Biodiet No. 1 and Skretting 0.6G. The experiment was terminated at day 58 post-hatch (35 days from the start of the experiment). Measurements of the weight and length of 20 fish from each tank were recorded at regular intervals during the experiment. Mortalities were recorded daily, and at the termination of the experiment, the survivors were counted and a random sample of 30 fish from each tank was individually weighed and measured.

### 6.2.5. Experiment 5. - Condition prior to weaning

#### (i) Live feed quality during larval rearing

*Trial 1*

This experiment was conducted in order to investigate the effect of larval feed quality on subsequent weaning success. Three batches of *R. tapirina* larvae were reared in 160 l tanks and fed with rotifers and *Artemia* enriched with either Frippak Booster, microalgae, or microalgae followed by Frippak Booster, as described in section 5.2.4. (iii). At day 33 post-hatch, three batches of 250 fish from each 160 l tank were counted into three randomly selected tanks out of the nine in the 25 l system. Initial lengths and weights are given in Table 6.2.5.

**Table 6.2.5. Initial lengths and weights ($\bar{x} \pm$ s.e., n=50 fish) of fish used in Trial 1.**

| Group | Enrichment | Length (mm) | Weight (g) |
|---|---|---|---|
| 1 | Frippak | 12.4 (0.2) | 0.025 (0.001) |
| 2 | Microalgae | 11.8 (0.2) | 0.021 (0.001) |
| 3 | Microalgae (15 days) then Frippak Booster | 11.9 (0.2) | 0.220 (0.011) |

The weaning phase was initiated from day 33 post-hatch using a 10 day schedule. The artificial diet used was a 1:1 mix of Biodiet No. 1 and Skretting 0.6G. All fish were fed on the same enriched *Artemia* they had been fed on prior to being removed from the 160 l system. The experiment was terminated at day 84 post-hatch (51 days from the start of the experiment in the 25 l system). Measurements of the weight and length of 20 fish from each tank were recorded at regular intervals during the experiment. Mortalities were recorded daily, and at the termination of the experiment, the survivors were counted and a random sample of 30 fish from each tank was individually weighed and measured.

*Trial 2*

Experiment 5, Trial 1 was repeated in 1993. Three batches of *R. tapirina* larvae were reared in 160 l tanks and fed with rotifers and *Artemia* enriched with either Frippak Booster, Nutri-Pack, or microalgae followed by Frippak Booster, as described in section 5.2.4. (iv). Initial lengths and weights are given in Table 6.2.6. These fish were

much smaller than those used in previous trials due to poor growth during the larval period.

**Table 6.2.6. Initial lengths and weights ($\bar{x} \pm$ s.e., n=50 fish) of fish used in Trial 2.**

| Group | Enrichment | Length (mm) | Weight (g) |
|---|---|---|---|
| 1 | Frippak | 6.92 (0.2) | 0.0052 (0.0004) |
| 2 | Nutri-Pack | 7.24 (0.2) | 0.0065 (0.0004) |
| 3 | Microalgae (15 days) then Frippak Booster | 7.25 (0.2) | 0.0052 (0.0004) |

The weaning phase was initiated from day 33 post-hatch using a 10 day schedule. The artificial diet used was a 1:1 mix of Biodiet No. 1 and Skretting 0.6G. All fish were fed on the same enriched *Artemia* they had been fed on prior to being removed from the 160 l system. The experiment was terminated at day 76 post-hatch (43 days from the start of the experiment in the 25 l system). Measurements of the weight and length of 20 fish from each tank were recorded at regular intervals during the experiment,. Mortalities were recorded daily, and at the termination of the experiment, the survivors were counted and a random sample of 30 fish from each tank was individually weighed and measured.

**(ii) Feed rate prior to weaning**

An experiment was conducted in order to investigate the effect of partial starvation of the larvae on subsequent weaning success. This was carried out by feeding with different feed rates of *Artemia* prior to weaning. Each tank was stocked with 250 (10/l) juvenile flounder of 33 days post-hatch (length = 13.9 ± 0.3 mm, weight = 0.034 ± 0.002 g; $\bar{x} \pm$ s.e., n=30 fish) previously fed on rotifers and *Artemia* enriched with microalgae (Appendix 1.2.). Each batch of fish was fed twice daily at a rate of either 1, 5 or 10 *Artemia* /ml/d. After a 10 day period of feeding at the set feed rates the weaning phase was initiated using the 20 day schedule but using the reduced rates of *Artemia* in proportion to those described above, for each treatment.

The artificial diet used was a 1:1 mix of Biodiet No. 1 and Skretting 0.6G. The experiment was terminated at day 79 post-hatch (46 days from the start of the experiment). Measurements of the weight and length of 20 fish from each tank were recorded at regular intervals during the experiment. Mortalities were recorded daily, and at the termination of the experiment, the survivors were counted and a random sample of 30 fish from each tank was individually weighed and measured.

## 6.3. RESULTS

### 6.3.1. Experiment 1. - Weaning diets

The artificial diet used for weaning did not have a great effect on growth, although survival was significantly ($P<0.05$) lower with Sevbar (Tables 6.3.1. and 6.3.2). Growth rates increased rapidly after weaning (Figs. 6.3.1. and 6.3.2.). There were no significant differences ($P>0.05$) in final length or weight between treatments. All survival rates were very poor compared with those obtained in the following experiments undertaken later in the study. The survival rate of fish weaned on Sevbar was significantly lower ($P<0.05$) than that of fish weaned on either Lansy or Biodiet/Skretting (Fig 6.3.3.). The main period of mortality occurred 5-10 days after the last *Artemia* was given.

**Table 6.3.1. Final lengths, weights and survival of three groups of *R. tapirina* fed with different artificial diets during weaning ($\bar{x} \pm$ s.e., n=3 replicates).**

| Diet | Final length (mm) | Final weight (g) | Survival (%) |
|---|---|---|---|
| Lansy | 19.8 (0.5) | 0.130 (0.008) | 16 (3)$^a$ |
| Sevbar | 22.5 (0.8) | 0.154 (0.014) | 7 (1)$^b$ |
| Biodiet & Skretting | 20.2 (0.4) | 0.141 (0.008) | 19 (3)$^a$ |

Figures in the same column sharing a common superscript are not significantly different ($P>0.05$).

**Table 6.3.2. Results of one-way ANOVA comparing the final lengths, weights and survival (arc sine $\sqrt{}$ transformed data) of *R. tapirina* weaned on different artificial diets.**

| Source | DF | SS | MS | F Ratio | P Value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 8.507 | 4.253 | 2.7614 | **P>0.05** |
| Error | 6 | 9.242 | 1.540 | | |
| Total | 8 | 17.749 | | | |
| Final weight | | | | | |
| Model | 2 | 0.00063 | 0.00031 | 0.985 | **P>0.05** |
| Error | 6 | 0.00191 | 0.00038 | | |
| Total | 8 | 0.00254 | | | |
| Survival | | | | | |
| Model | 2 | 0.0348 | 0.0174 | 5.338 | **P<0.05** |
| Error | 6 | 0.0195 | 0.0033 | | |
| Total | 8 | 0.0543 | | | |

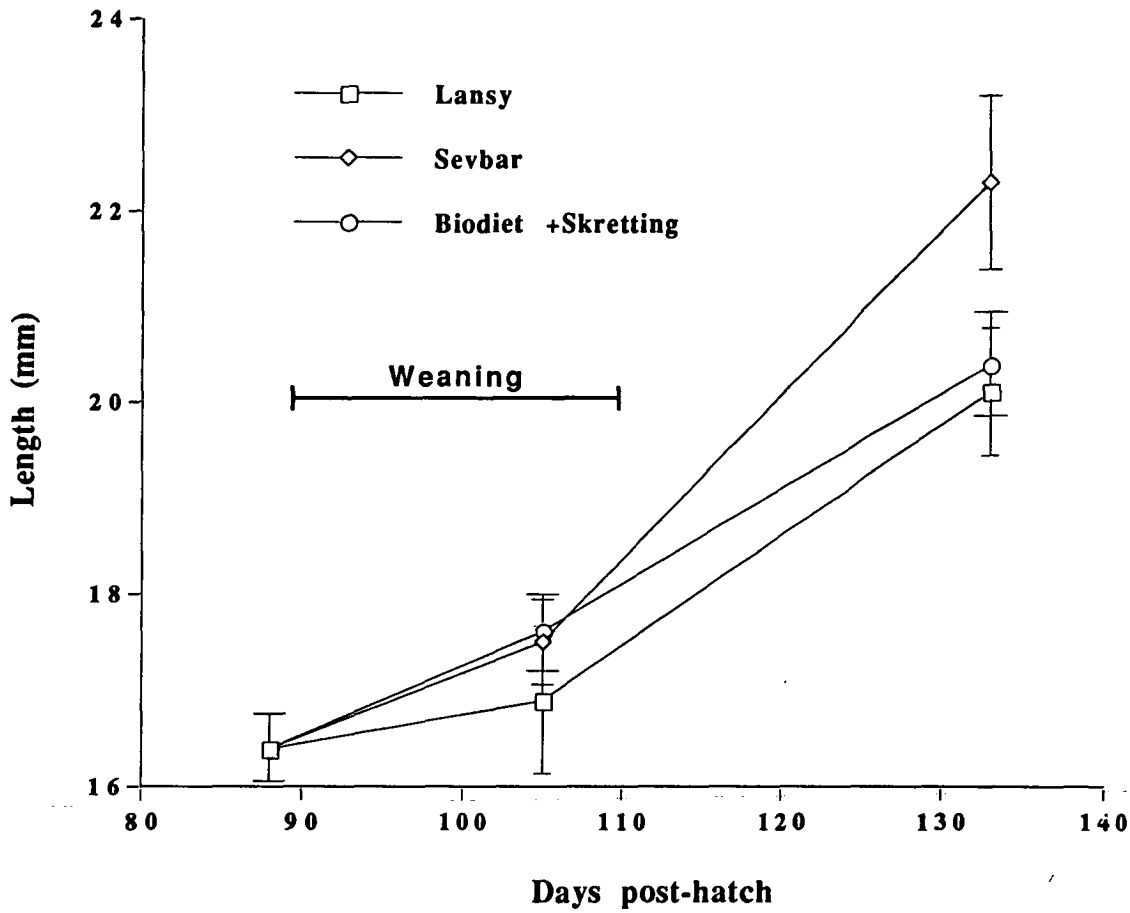


Fig. 6.3.1. Growth in length ($\bar{x} \pm$ s.e., n=3 replicates ) of *Rhombosolea tapirina* weaned on different artificial diets.

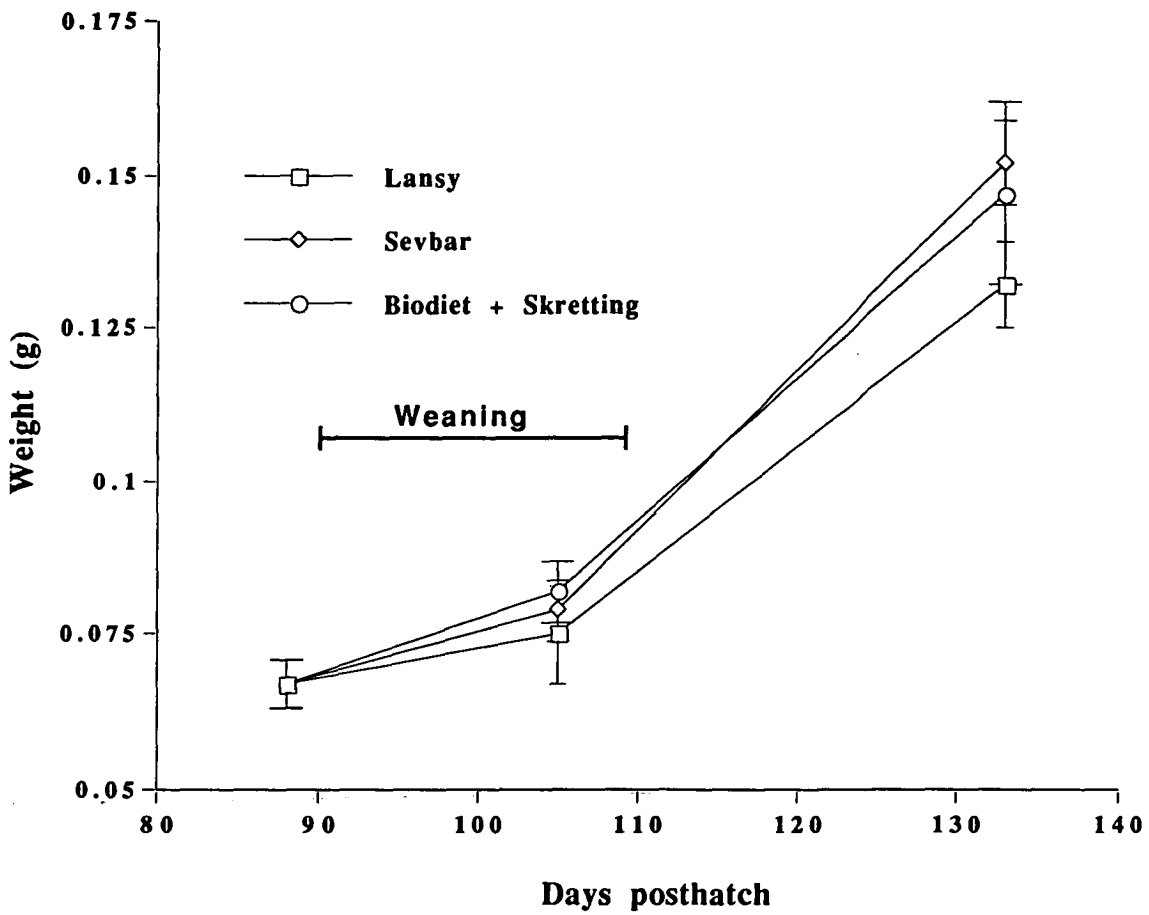


Fig. 6.3.2. Growth in weight ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned on different artificial diets.

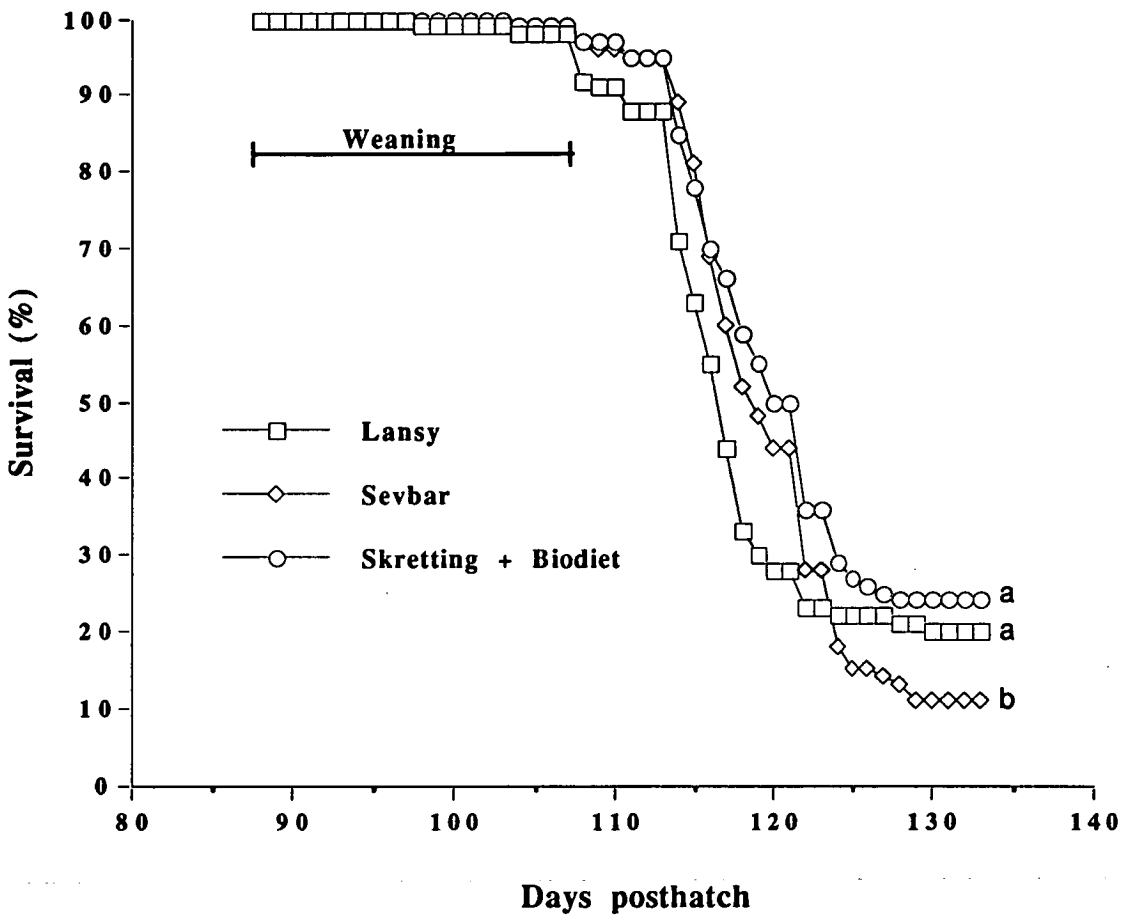


Fig. 6.3.3. Mean survival of *Rhombosolea tapirina* weaned on different artificial diets.

Survival is observed survival. Actual survival is given in Table 6.3.1

Maximum s.e. = ±7.08% (Lansy), ±7.91% (Skretting/Biodiet) and ±11.15% (Sevbar) over the experimental period.

Points sharing a common superscript are not significantly different (P>0.05).

### 6.3.2. Experiment 2. - Stocking density

The stocking density of *R. tapirina* had no effect on either growth or survival during weaning. There were no significant differences (P>0.05) between final mean lengths, weights or survival (Table 6.3.3. and 6.3.4). Growth rates were all very similar throughout the experiment (Figs. 6.3.4. and 6.3.5). The mortality rates showed a normal pattern, mortalities commencing approximately 6 days after the last *Artemia* and ending after approximately 14-20 days (Fig. 6.3.6).

**Table 6.3.3. Final lengths, weights and survival of three groups of *R. tapirina* weaned at different stocking densities ($\bar{x}$ ± s.e., n=3 replicates).**

| Stocking density | Final length (mm) | Final weight (g) | Survival (%) |
|---|---|---|---|
| 5 fish/l | 22.1 (0.3) | 0.175 (0.004) | 44.0 (3.3) |
| 10 fish/l | 21.5 (1.9) | 0.207 (0.015) | 47.2 (1.7) |
| 20 fish/l | 23.4 (0.7) | 0..222 (0.026) | 45.3 (7.7) |

There were no significant differences (P>0.05) between treatments.

**Table 6.3.4. Results of one-way ANOVA comparing the final lengths, weights and survival (arc sine √ transformed data) of *R. tapirina* weaned at different stocking densities.**

| Source | DF | SS | MS | F Ratio | P Value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 2.185 | 1.093 | 1.027 | **P>0.05** |
| Error | 6 | 6.384 | 1.064 | | |
| Total | 8 | 8.569 | | | |
| Final weight | | | | | |
| Model | 2 | 0.0026 | 0.0013 | 1.42 | **P>0.05** |
| Error | 6 | 0.0054 | 0.0009 | | |
| Total | 8 | 0.0079 | | | |
| Survival | | | | | |
| Model | 2 | 0.0016 | 0.0008 | 0.107 | **P>0.05** |
| Error | 6 | 0.0449 | 0.0075 | | |
| Total | 8 | 0.0465 | | | |

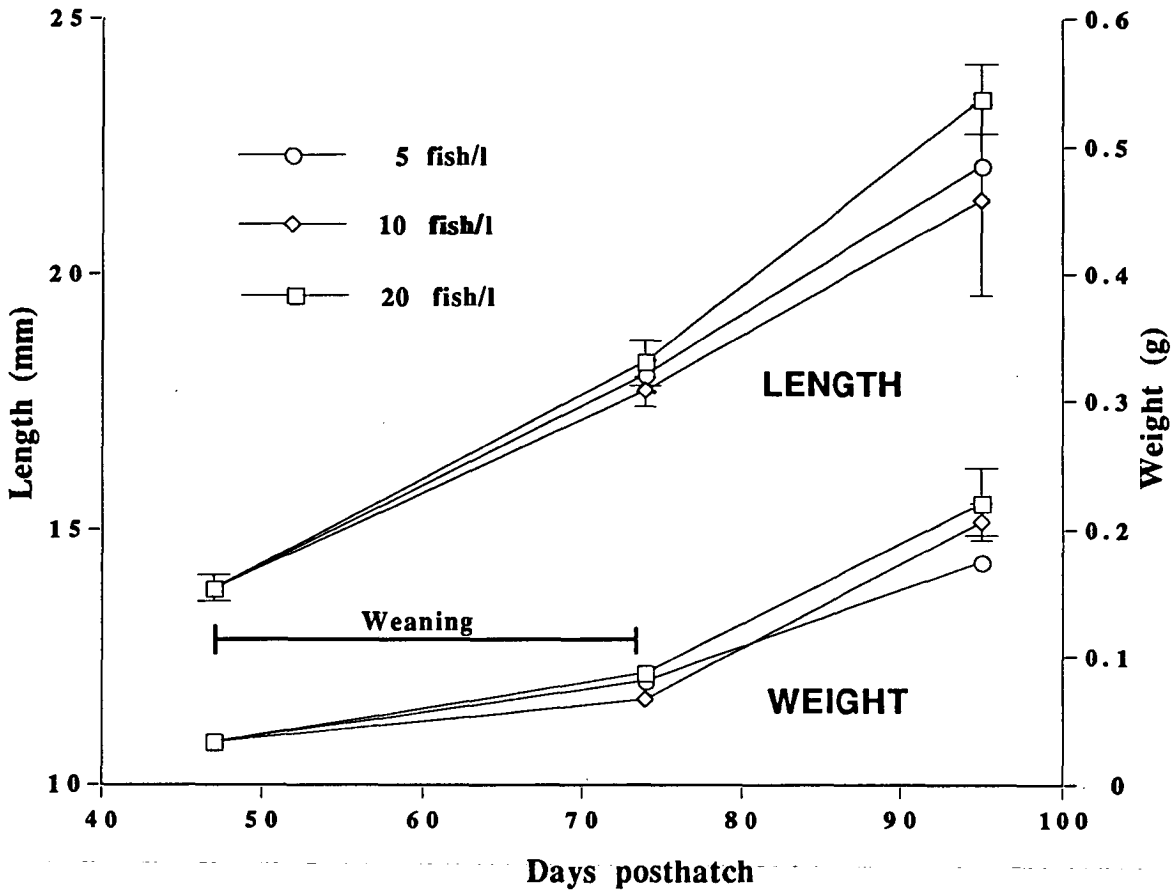


Fig. 6.3.4. Growth in weight and length ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned at different stocking densities.

Points with no visible error bars have very small s.e.

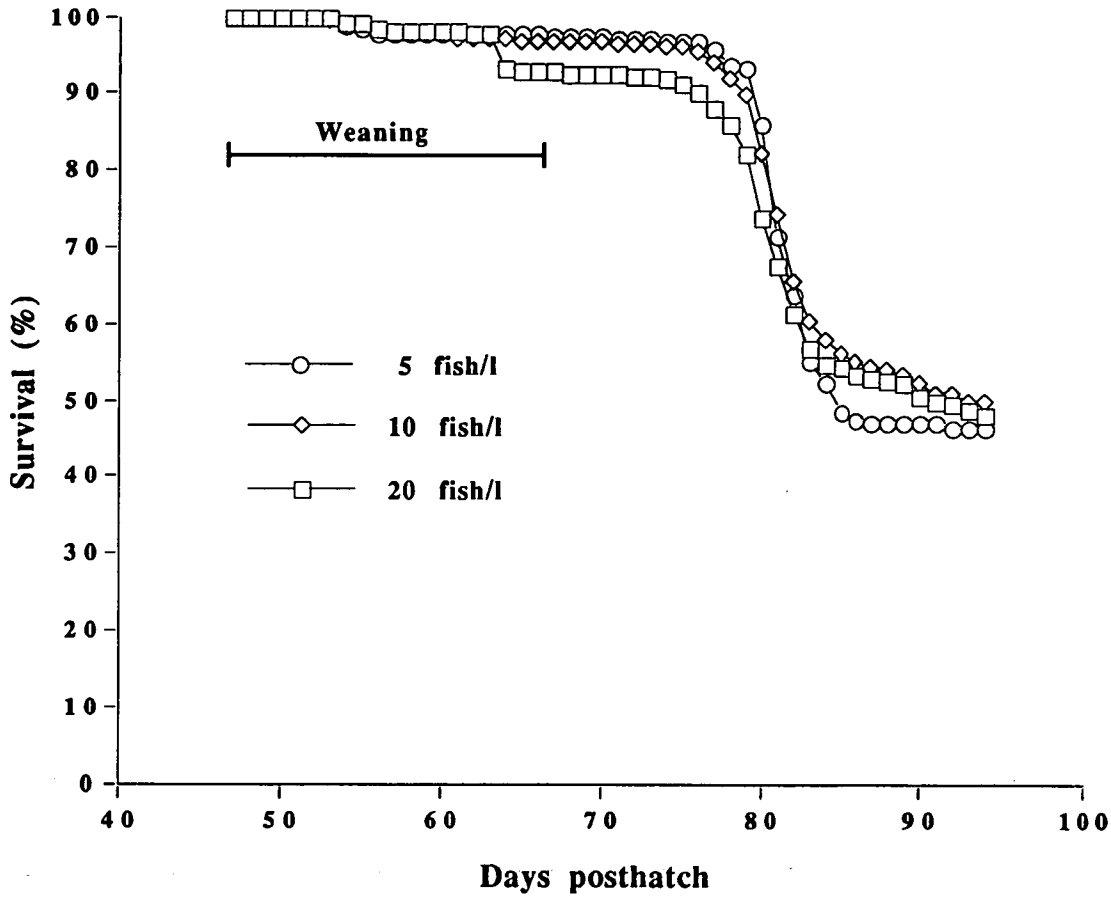


Fig. 6.3.5. Mean survival of *Rhombosolea tapirina* weaned at different stocking densities. Weaning was initiated at day 47 post-hatch for 20 days and the experiment was terminated on day 97 post-hatch.

Survival is observed survival. Actual survival is given in Table 6.3.4.

Maximum s.e. = ±5.3% (5 fish/l), ±3.06% (10 fish/l) and ±10.25% (20 fish/l) over the experimental period.

### 6.3.3. Experiment 3. - Length of the changeover period

The length of the weaning period had a considerable effect on growth with the more *Artemia* being fed (the longer weaning periods), the better the growth rate (Tables 6.3.5. and 6.3.6. and Figs. 6.3.6. and 6.3.7.). The final length and weight of fish weaned over a 20 or 10 day period was significantly greater ($P<0.05$) than those weaned over 5 days. However, the final mean weights and lengths of fish weaned over a 5 day period were lower than other treatments. Overall survival was very high in this experiment. There were no significant differences ($P>0.05$) in survival between treatments. Mortalities began to occur earlier in the fish weaned over a 5 day period but otherwise the rate of mortality was similar in all three treatments and fairly constant after the last *Artemia* (Fig. 6.3.8.).

**Table 6.3.5. Final lengths, weights and survival of *R. tapirina* weaned over different time periods ($\bar{x} \pm$ s.e., n=3 replicates).**

| Length of changeover. | Final length (mm) | Final weight (g) | Survival (%) |
|---|---|---|---|
| 5 days | 14.2 (0.3)$^a$ | 0.051 (0.004)$^a$ | 66.2 (5.5) |
| 10 days | 16.0 (0.3)$^b$ | 0.071 (0.004)$^b$ | 73.8 (2.8) |
| 20 days | 17.9 (0.4)$^b$ | 0.104 (0.007)$^b$ | 68.7 (4.1) |

Figures in the same column sharing a common superscript are not significantly different ($P>0.05$).

**Table 6.3.6. Results of one-way ANOVA comparing the final lengths (1/Y transformed data due to +ve skewed distribution), weights (1/√Y transformed data due to +ve skewed distribution) and survival (arc sine √ transformed data) of *R.tapirina* weaned over different time periods.**

| Source | DF | SS | MS | F Ratio | P Value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 0.00034 | 0.00017 | 14.012 | **P<0.05** |
| Error | 6 | 0.000072 | 0.000012 | | |
| Total | 8 | 0.000407 | | | |
| Final weight | | | | | |
| Model | 2 | 2.82204 | 1.411 | 11.784 | **P<0.05** |
| Error | 6 | 0.71842 | 0.1197 | | |
| Total | 8 | 3.54046 | | | |
| Survival | | | | | |
| Model | 2 | 0.0106 | 0.0053 | 0.819 | **P>0.05** |
| Error | 6 | 0.0390 | 0.0065 | | |
| Total | 8 | 0.0496 | | | |

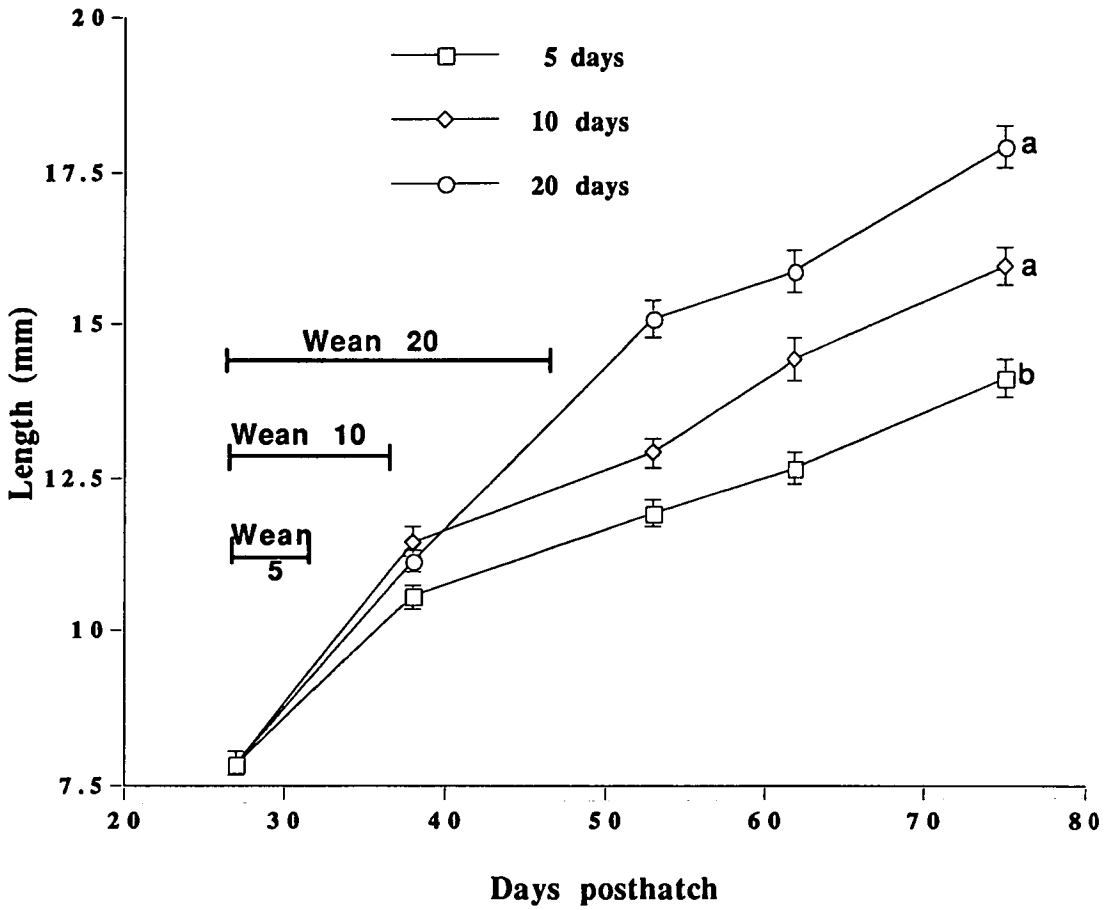


Fig. 6.3.6. Growth in length ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned over 5, 10 or 20 days.

Points sharing a common superscript are not significantly different (P>0.05)

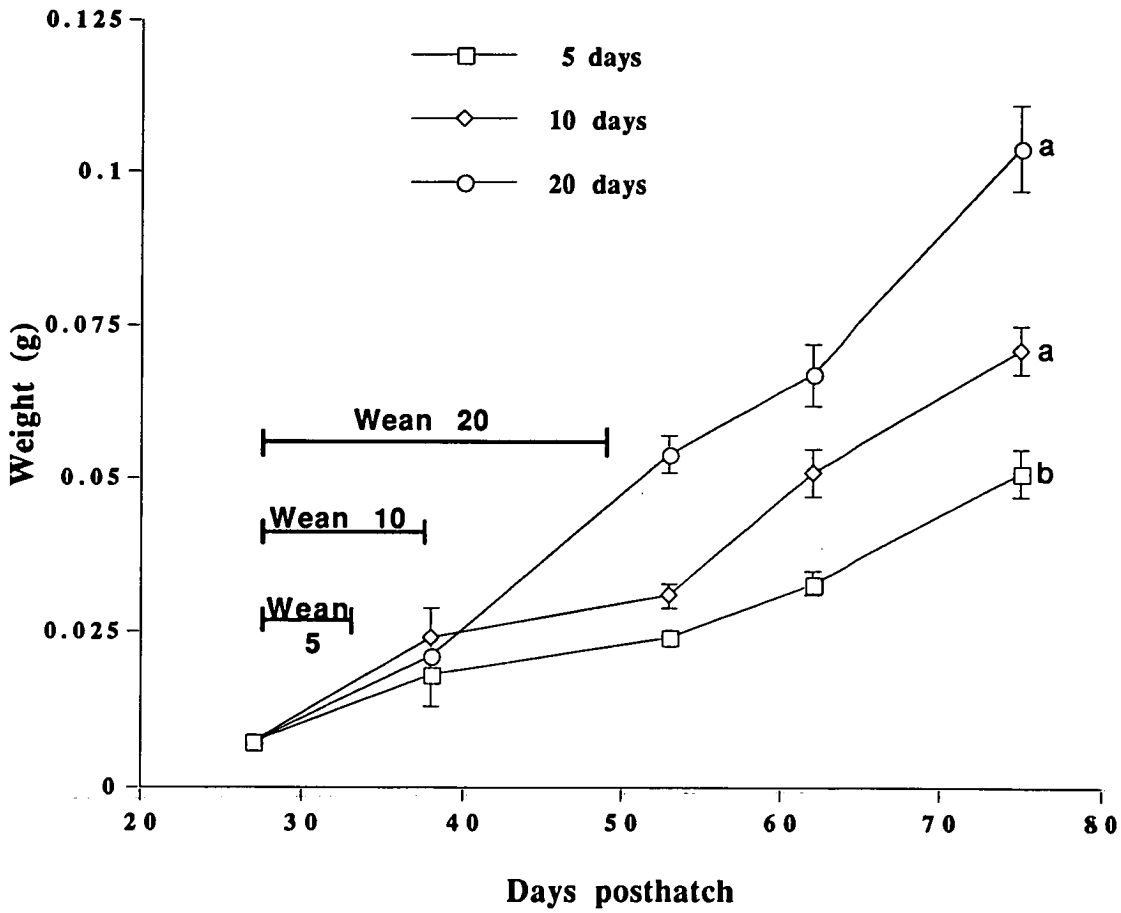


Fig. 6.3.7. Growth in weight ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned over 5, 10 or 20 days.

Points sharing a common superscript are not significantly different (P>0.05)

Points with no visible error bars have very small s.e.

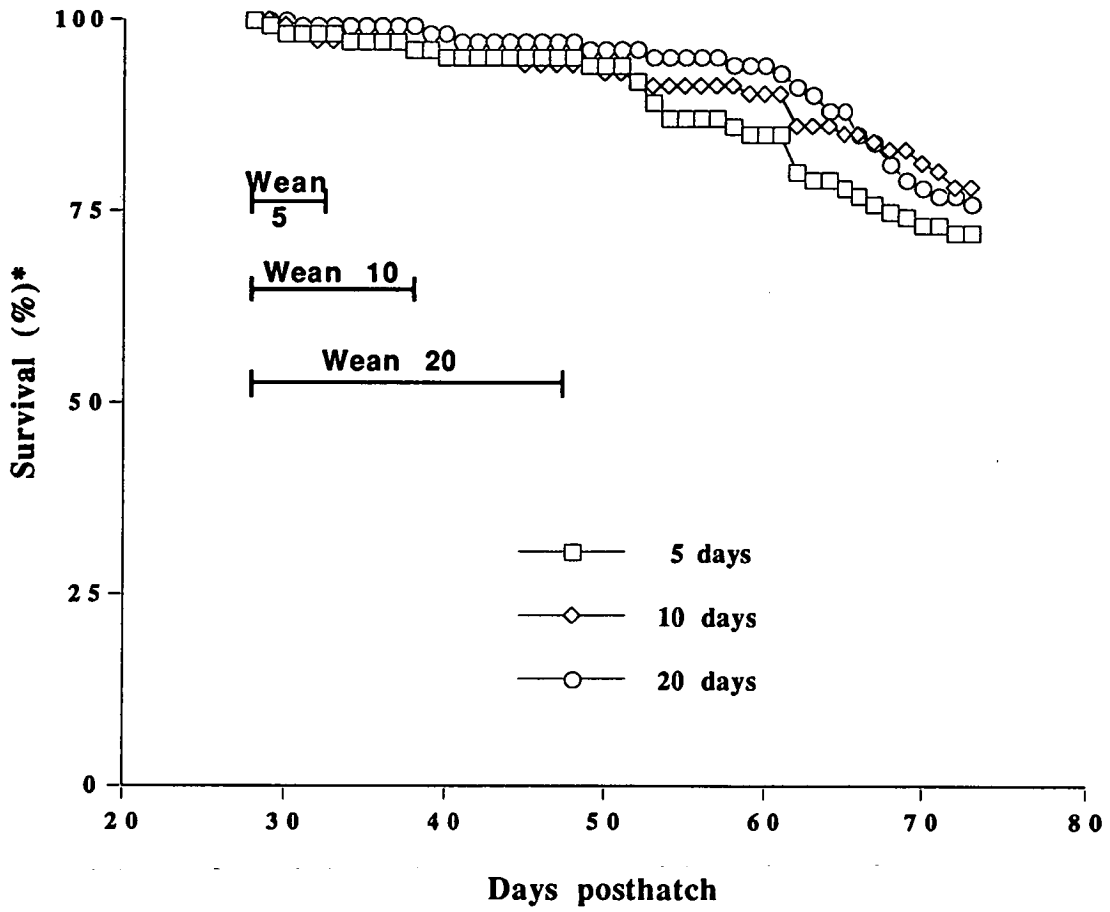


Fig. 6.3.8. Mean survival rates of *Rhombosolea tapirina* weaned over 5, 10 or 20 days.

Survival is observed survival. Actual survival is given in Table 6.3.5.

* Maximum s.e. = ±5.24% (5 day weaning), ±2.93% (10 day weaning) and ±2.67% (20 day weaning) over the experimental period.

### 6.3.4. Experiment 4. - Age and size at weaning

#### (i) Weaning at days 42, 50 and 58

The result of weaning at different ages and sizes was compared by weaning batches from the same group of fish, at different days post-hatch. Earlier weaning resulted in better survival rates. There were no significant differences ($P > 0.05$) in final mean lengths or weights between treatments (Table 6.3.7. and 6.3.8). Total mortality occurred in one replicate from group 2, on day 37 of the experiment, due to blockage of the inlet pipe. This replicate was therefore excluded from the analysis of survival rates. Growth rates were fairly constant throughout the experiment with the exception of group 2 which showed a reduction in the growth rate during the weaning period (Figs 6.3.9. and 6.3.10.). This may have been due to fin-biting reducing the lengths and poor condition reducing the weight or it may have been an unrepresentative sample taken on day 54 post-hatch. The survival rate of group 1 fish was significantly ($P < 0.05$) greater than that of group 3. Mortality rates all showed a normal pattern with mortalities commencing 5 days after the last *Artemia* and ending 14-20 days later (Fig 6.3.11.).

**Table 6.3.7. Final lengths, weights and survival of *R. tapirina* weaned at different days post-hatch ($\bar{x} \pm$ s.e., n=3 replicates).**

| Age at which weaning commenced | Final length (mm) | Final weight (g) | Survival (%) |
|---|---|---|---|
| Group 1. 42 days post-hatch | 26.7 (0.4) | 0.295 (0.009) | 58.2 (3.8)$^{a}$ |
| Group 2. 50 days post-hatch | 27.1 (0.3) | 0.283 (0.031) | 50.9 (6.5)$^{ab}$ |
| Group 3. 58 days post-hatch | 27.4 (0.3) | 0.294 (0.013) | 38.1 (2.7)$^{b}$ |

Figures in the same column sharing a common superscript are not significantly different ($P > 0.05$).

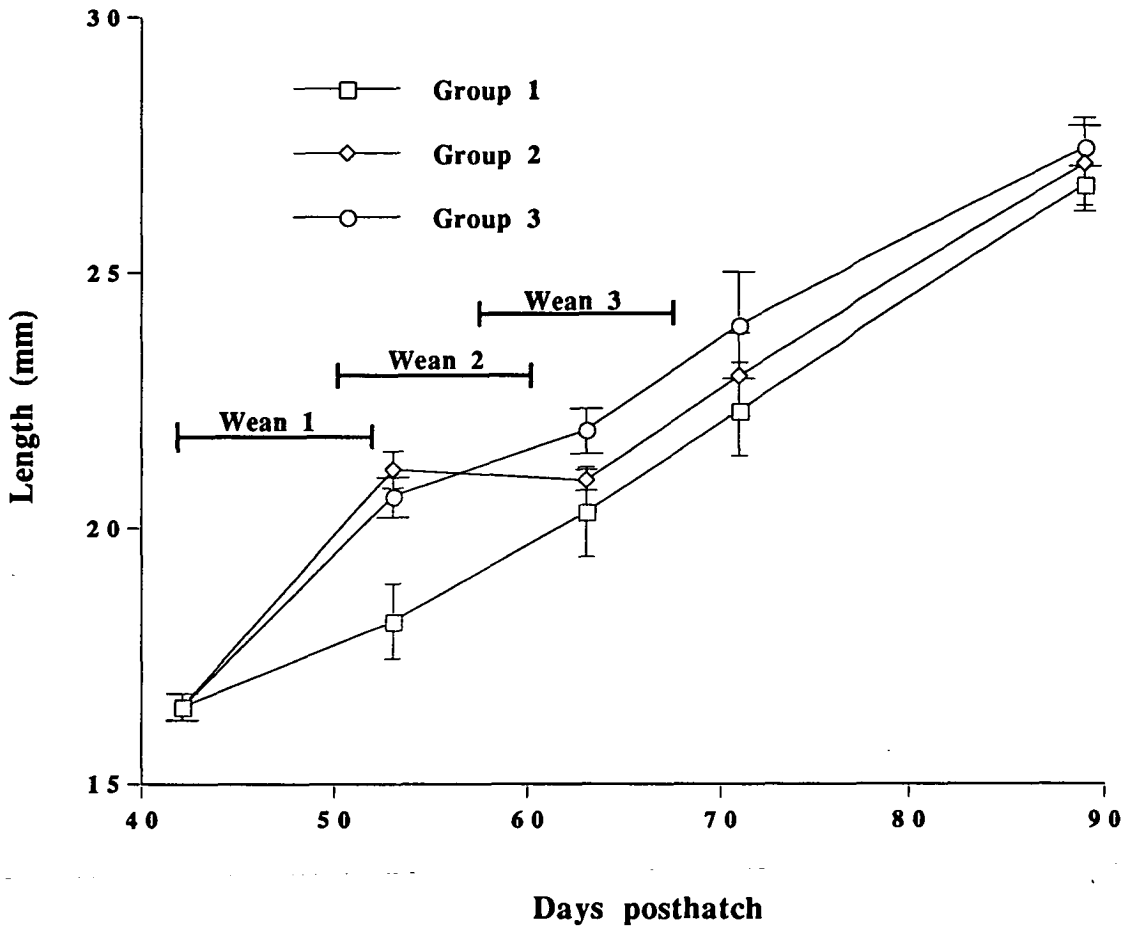


Fig. 6.3.9. Growth in length ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned at days 42 (Group 1), 50 (Group 2) or 58 (Group 3) days post-hatch.

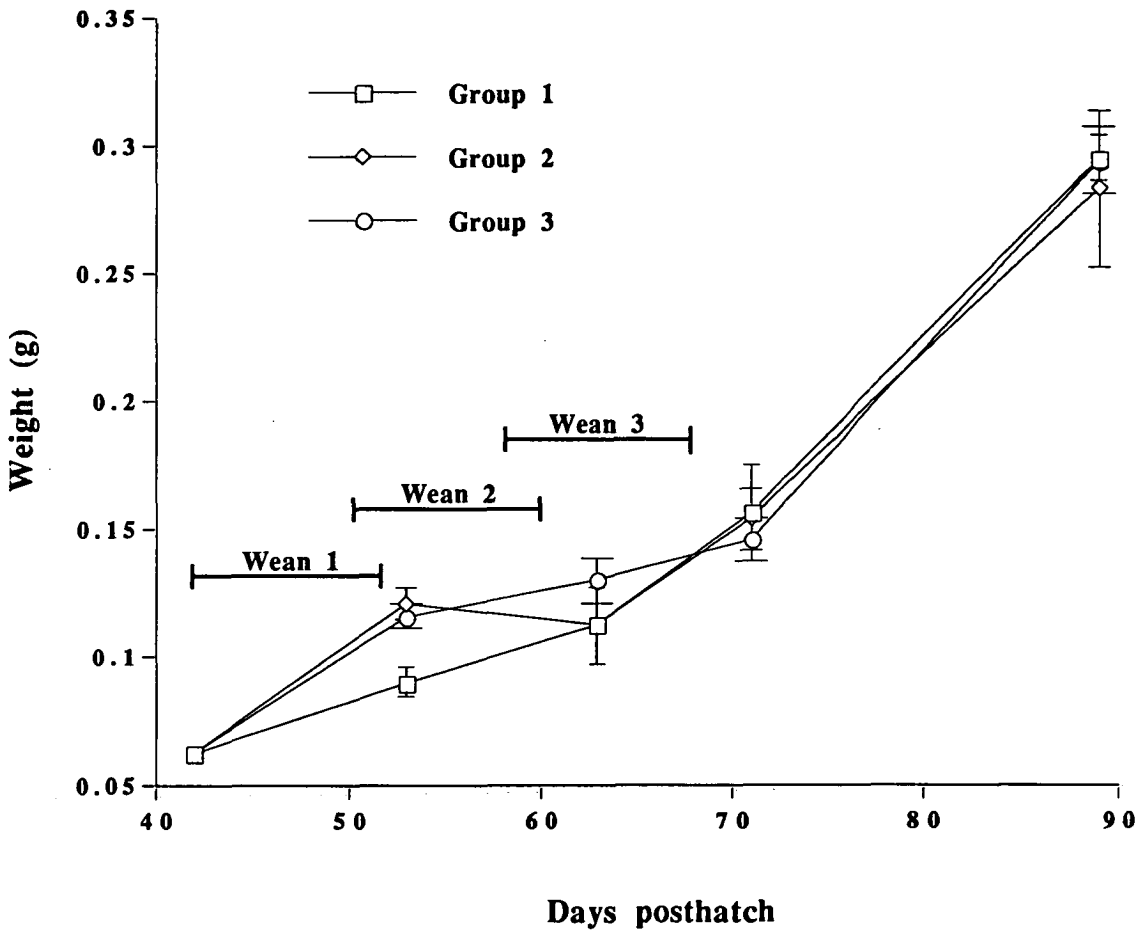


Fig. 6.3.10. Growth in weight ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned at days 42 (Group 1), 50 (Group 2) or 58 (Group 3) days post-hatch.

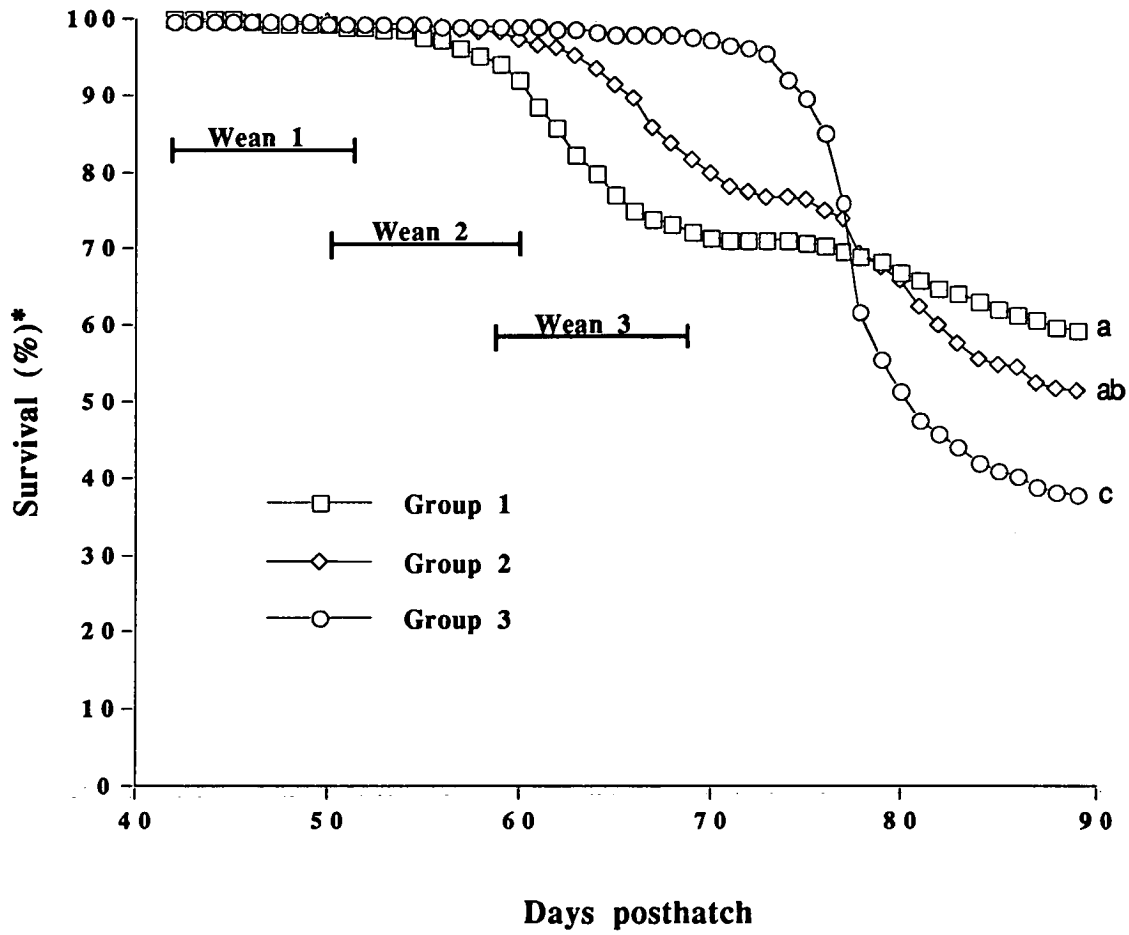


Fig 6.3.11. Mean survival of *Rhombosolea tapirina* weaned at days 42 (Group 1), 50 (Group 2) or 58 (Group 3) days post-hatch.

Points sharing a common superscript are not significantly different (P>0.05)

* Survival is observed survival,. Actual survival is given in Table 6.3.7

Maximum s.e. = ±4.35% (group 1), ±7.19% (group 2) and ±5.95% (group 3) over the experimental period.

**Table 6.3.8. Results of one-way ANOVA comparing the final lengths, weights and survival (arc sine √ transformed data) of *R. tapirina* weaned at different days post-hatch.**

| Source | DF | SS | MS | F Ratio | P Value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 0.856 | 0.43 | 0.602 | **P>0.05** |
| Error | 5 | 3.571 | 0.71 | | |
| Total | 7 | 4.431 | | | |
| Final weight | | | | | |
| Model | 2 | 0.00018 | 0.00009 | 0.137 | **P>0.05** |
| Error | 5 | 0.00333 | 0.00067 | | |
| Total | 7 | 0.00351 | | | |
| Survival | | | | | |
| Model | 2 | 0.0638 | 0.0319 | 7.024 | **P<0.05** |
| Error | 5 | 0.0227 | 0.0046 | | |
| Total | 7 | 0.0866 | | | |

**(ii) Weaning before metamorphosis**

Weaning at day 23 post-hatch resulted in higher survival rates than recorded in any other weaning experiments (Table 6.3.9.). The daily mortality showed a slight increase at approximately 15 days after the last *Artemia*, but it remained lower than recorded in any other weaning experiments (Fig. 6.3.13.). Growth rate in weight increased after weaning, but the rate of increase in length showed little change during the experiment (Fig. 6.3.12.). Although there was no control used in this experiment it shows that weaning can be carried out before metamorphosis and a tentative comparison can be made to other weaning experiments in which the weaning period was initiated at a later stage of development.

**Table 6.3.9. Weight, length and survival of *R. tapirina* weaned at day 23 post-hatch ($\bar{x} \pm$ s.e., n=3 replicates).**

| Days post-hatch | Length (mm) | Weight (g) |
|---|---|---|
| 37 | 11.9 (0.3) | 0.0188 (0.0013) |
| 57 | 14.9 (0.1) | 0.0445 (0.0018) |
| Survival (%) | 82.2 (4.3) | |

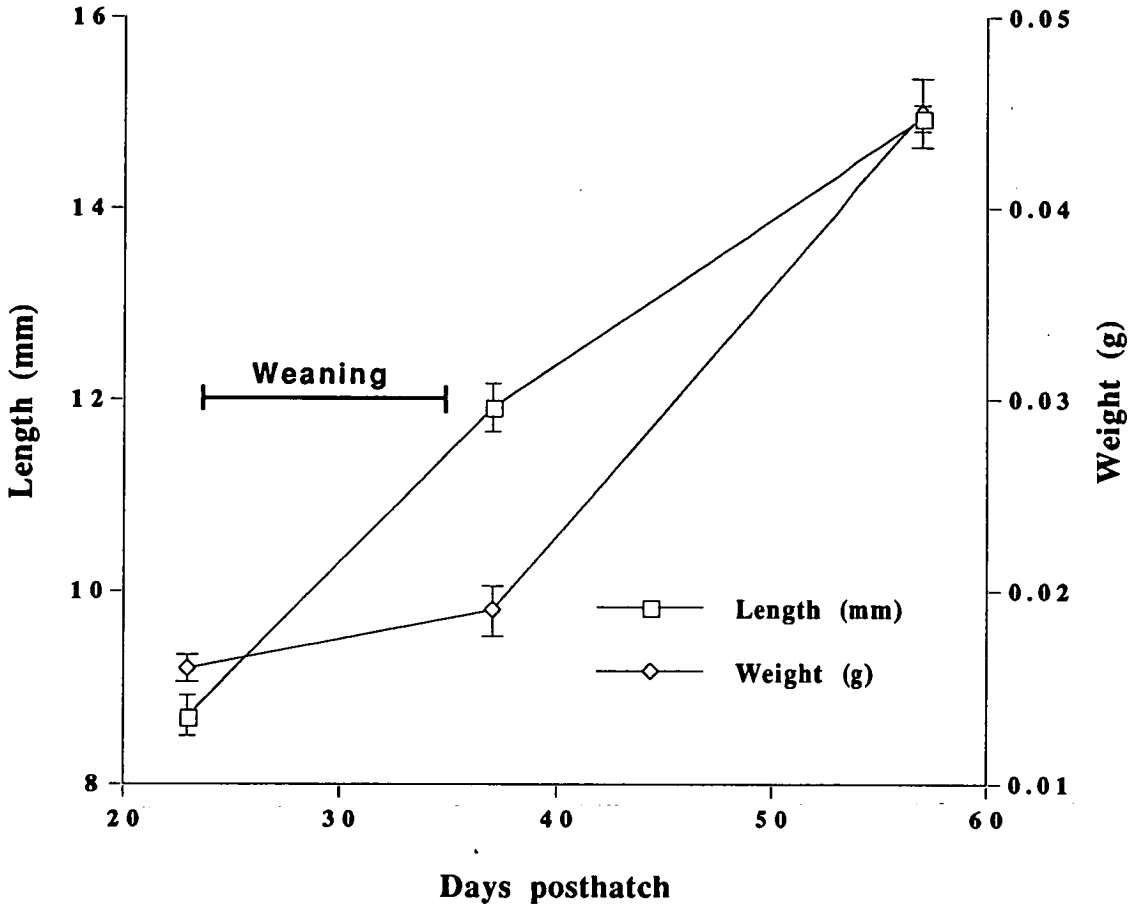


Fig. 6.3.12. Growth in weight and length ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned at day 23 post-hatch.

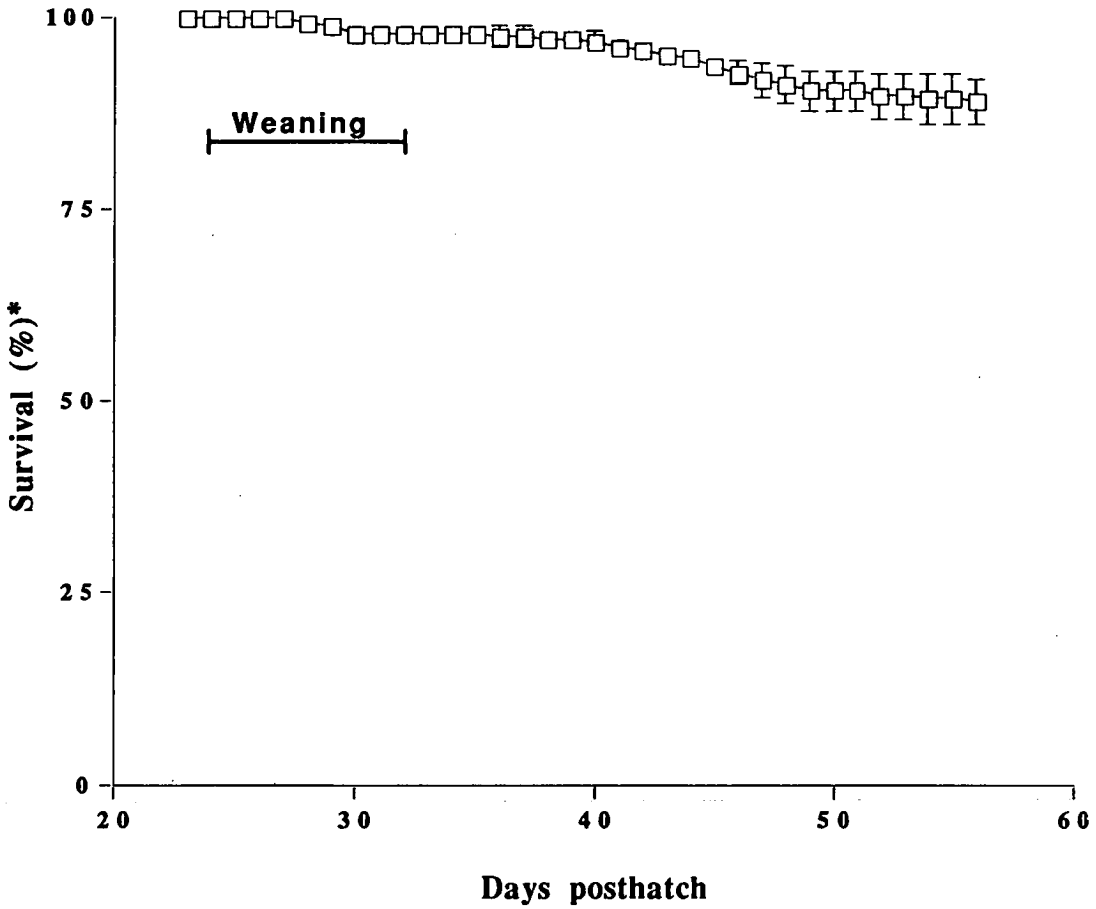


Fig. 6.3.13. Survival ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned at day 23 post-hatch.

* Survival is observed survival. Actual survival is given in Table 6.3.9.

### 6.3.5. Experiment 5. - Condition prior to weaning

#### (i) Live feed quality during larval rearing

*Trial 1*

Enrichment of live feeds had a considerable effect on growth and survival rates of flounder during weaning. The final mean length and weight of group 3 was significantly ($P< 0.05$) greater than that of groups 1 and group 2. The final mean length and weight of group 1 was significantly ($P< 0.05$) greater than that of group 2 (Tables 6.3.10. and 6.3.11). Growth rates in all treatments increased considerably at approximately 25 days after the last *Artemia* were introduced (Figs. 6.3.14. and 6.3.15.).

The survival rate of group 2 was significantly ($P<0.05$) lower than that of groups 1 and 3. The rate of mortality showed the normal pattern with the main mortality commencing approximately 6 days after the last *Artemia* had been introduced and ending after approximately 9 days (Fig. 6.3.16.). Enrichment with Algae followed by Frippak after 15 days, resulted in improved growth and survival as compared to Frippak or algae. Algae alone resulted in the lowest survival and growth rates.

**Table 6.3.10. Final lengths, weights and survival of *R. tapirina* weaned after initial feeding with live feeds enriched with different diets ($\bar{x} \pm$ s.e., n=3 replicates).**

| Enrichment used for live feeds. | Final length (mm) | Final weight (g) | Survival (%) |
|---|---|---|---|
| Group 1. Frippak | 23.23 (0.215)$^a$ | 0.206 (0.007)$^a$ | 58.13 (2.68)$^a$ |
| Group 2. Algae | 20.42 (0.205)$^b$ | 0.131 (0.007)$^b$ | 47.87 (1.96)$^b$ |
| Group 3. Algae for 15 days then Frippak | 25.09 (0.692)$^c$ | 0.266 (0.023)$^c$ | 60.93 (2.76)$^a$ |

Figures in the same column sharing a common superscript are not significantly different ($P>0.05$).

**Table 6.3.11. Results of one-way ANOVA comparing the final lengths, weights and survival (arc sine √ transformed data) of *R. tapirina* weaned after feeding with live feeds enriched with different diets.**

| Source | DF | SS | MS | F Ratio | P Value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 33.071 | 16.536 | 29.21 | **P<0.01** |
| Error | 6 | 3.397 | 0.5661 | | |
| Total | 8 | 36.468 | | | |
| Final weight | | | | | |
| Model | 2 | 0.0272 | 0.0136 | 21.152 | **P<0.01** |
| Error | 6 | 0.0039 | 0.0006 | | |
| Total | 8 | 0.0311 | | | |
| Survival | | | | | |
| Model | 2 | 0.0289 | 0.0144 | 7.557 | **P>0.05** |
| Error | 6 | 0.0115 | 0.0019 | | |
| Total | 8 | 0.0404 | | | |

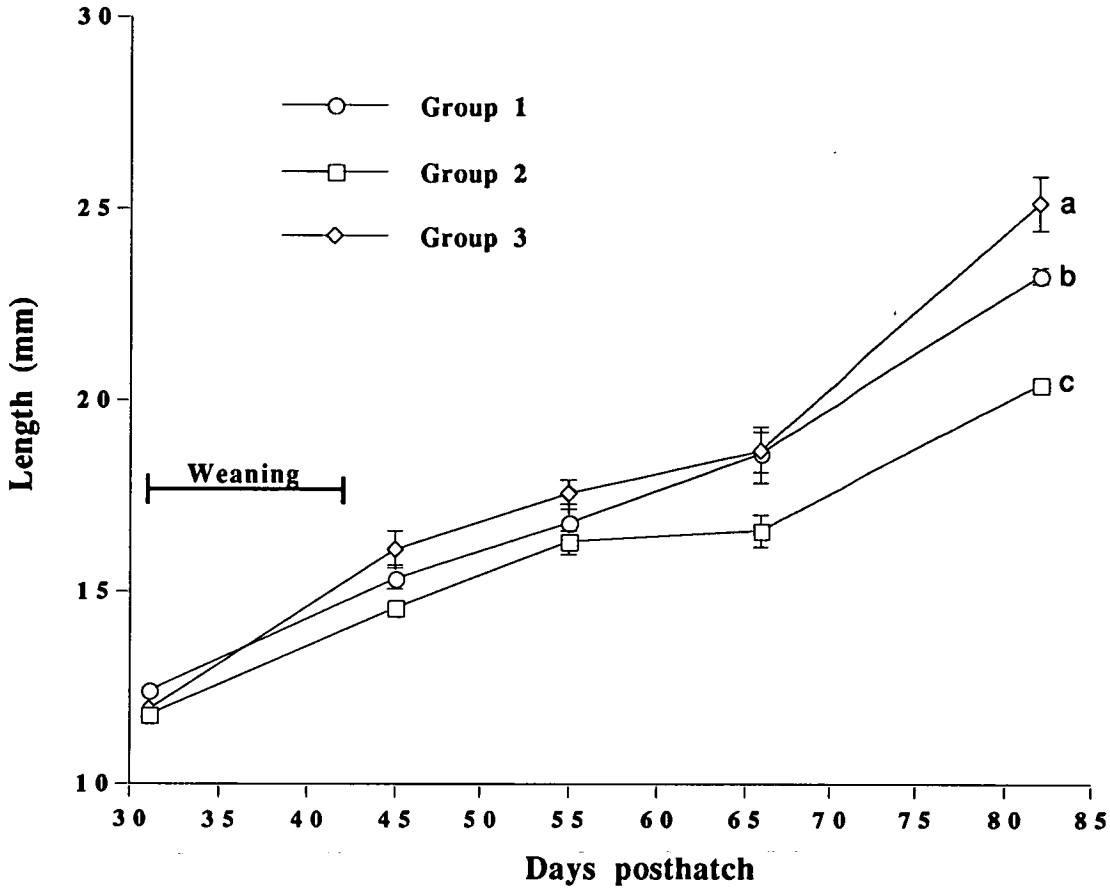


Fig. 6.3.14. Growth in length ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned after feeding on live feeds enriched with Frippak (Group 1), microalgae (Group 2) or microalgae followed by Frippak after day 15 post-hatch (Group 3).

Points sharing a common superscript are not significantly different (P>0.05)

Points with no visible error bars have very small s.e.

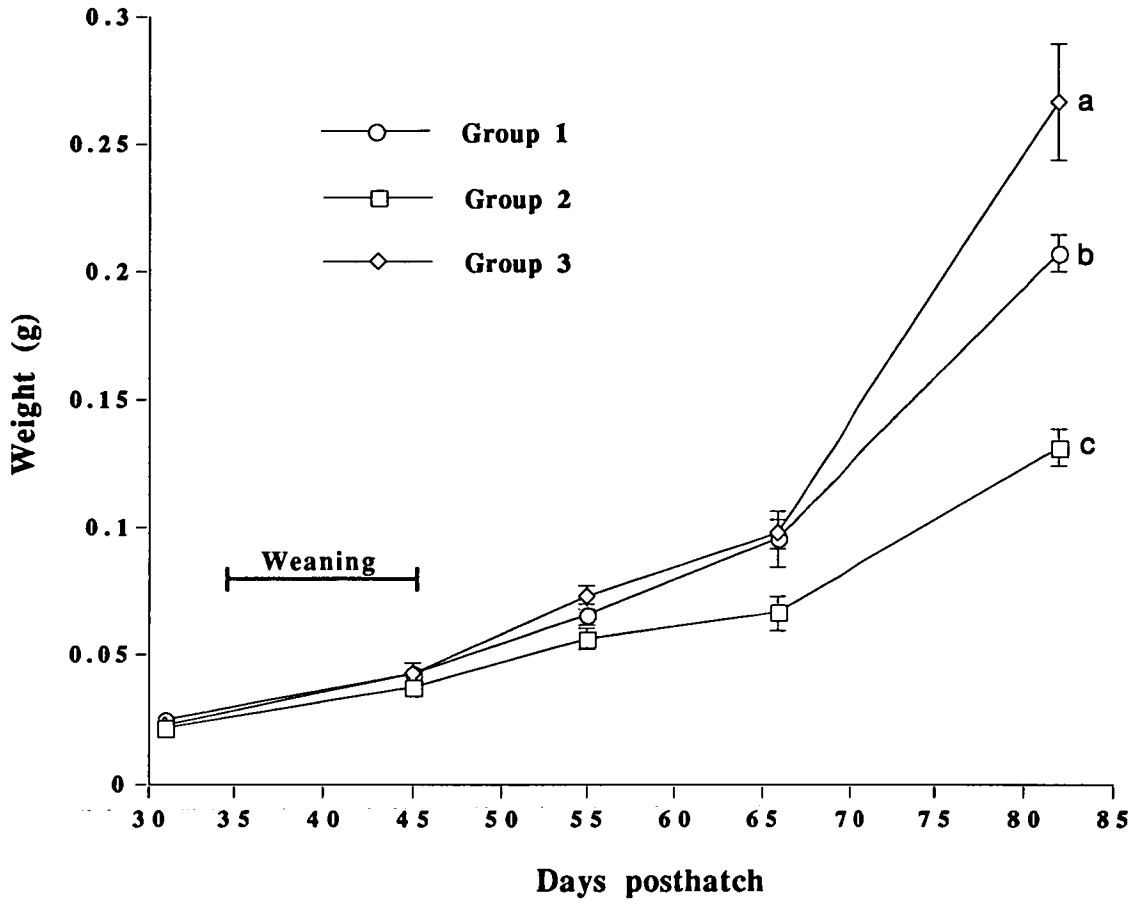


Fig. 6.3.15. Growth in weight ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned after feeding on live feeds enriched with Frippak (Group 1), microalgae (Group 2) or microalgae followed by Frippak after day 15 post-hatch (Group 3).

Points sharing a common superscript are not significantly different (P>0.05)

Points with no visible error bars have very small s.e.

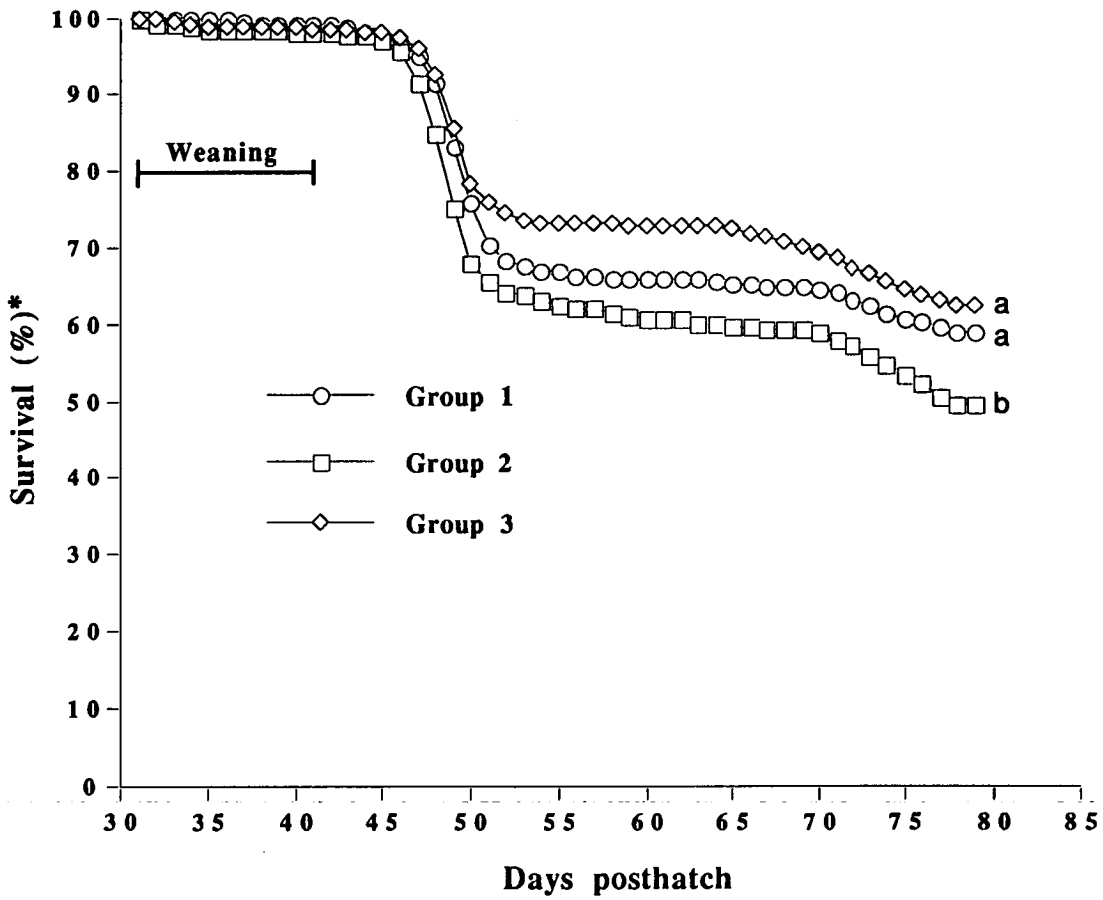


Fig. 6.3.16. Mean survival rates of *Rhombosolea tapirina* weaned after feeding on live feeds enriched with Frippak (Group 1), microalgae (Group 2) or microalgae followed by Frippak after day 15 post-hatch (Group 3).

Points sharing a common superscript are not significantly different (P>0.05)

Maximum s.e.±2.51% (Frippak), ±5.09% (algae) and ±4.46% (algae + Frippak) over the experimental period.

*survival is observed survival rather than actual survival as shown in Table 6.3.10

*Trial 2*

Enrichment of live feeds had no effect on either growth or survival rates of *R. tapirina* through weaning in this experiment. There were no significant differences (P>0.05) in weight, length or survival between treatments (Tables 6.3.12. and 6.3.13). Growth rates were considerably reduced after weaning and were generally poor compared to the previous experiment (Figs. 6.3.17., 6.3.18.). Mortality showed the normal pattern, mortality occurring at approximately 6 days after the last *Artemia* and ending around 7 days later. Survival was lower than recorded in other weaning experiments (Fig. 6.3.19.).

**Table 6.3.12. Final lengths, weights and survival of *R. tapirina* weaned after initial feeding with live feeds enriched with different diets ($\bar{x} \pm$ s.e., n=3 replicates).**

| Enrichment used for live feeds. | Final length (mm) | Final weight (g) | Survival (%) |
|---|---|---|---|
| Group 1. Frippak | 13.6 (0.3) | 0.042 (0.003) | 36.4 (5.9) |
| Group 2. Algae | 13.5 (0.2) | 0.040 (0.002) | 32.8 (9.2) |
| Group 3. Nutri-Pack | 13.6 (0.3) | 0.042 (0.003) | 35.9 (7.8) |

There were no significant differences (P>0.05) between treatments.

**Table 6.3.13. Results of one-way ANOVA comparing the final lengths, weights and survival (arc sine $\sqrt{}$ transformed data) of *R. tapirina* weaned after feeding with live feeds enriched with different diets.**

| Source | DF | SS | MS | F Ratio | P Value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 0.01069 | 0.0053 | 0.110 | **P>0.05** |
| Error | 6 | 0.29047 | 0.0484 | | |
| Total | 8 | 0.30116 | | | |
| Final weight | | | | | |
| Model | 2 | 0.0000183 | 0.000009 | 1.253 | **P>0.05** |
| Error | 6 | 0.0000438 | 0.000007 | | |
| Total | 8 | 0.0000621 | | | |
| Survival | | | | | |
| Model | 2 | 0.000018 | 0.000009 | 1.253 | **P>0.05** |
| Error | 6 | 0.000044 | 0.000007 | | |
| Total | 8 | 0.000062 | | | |

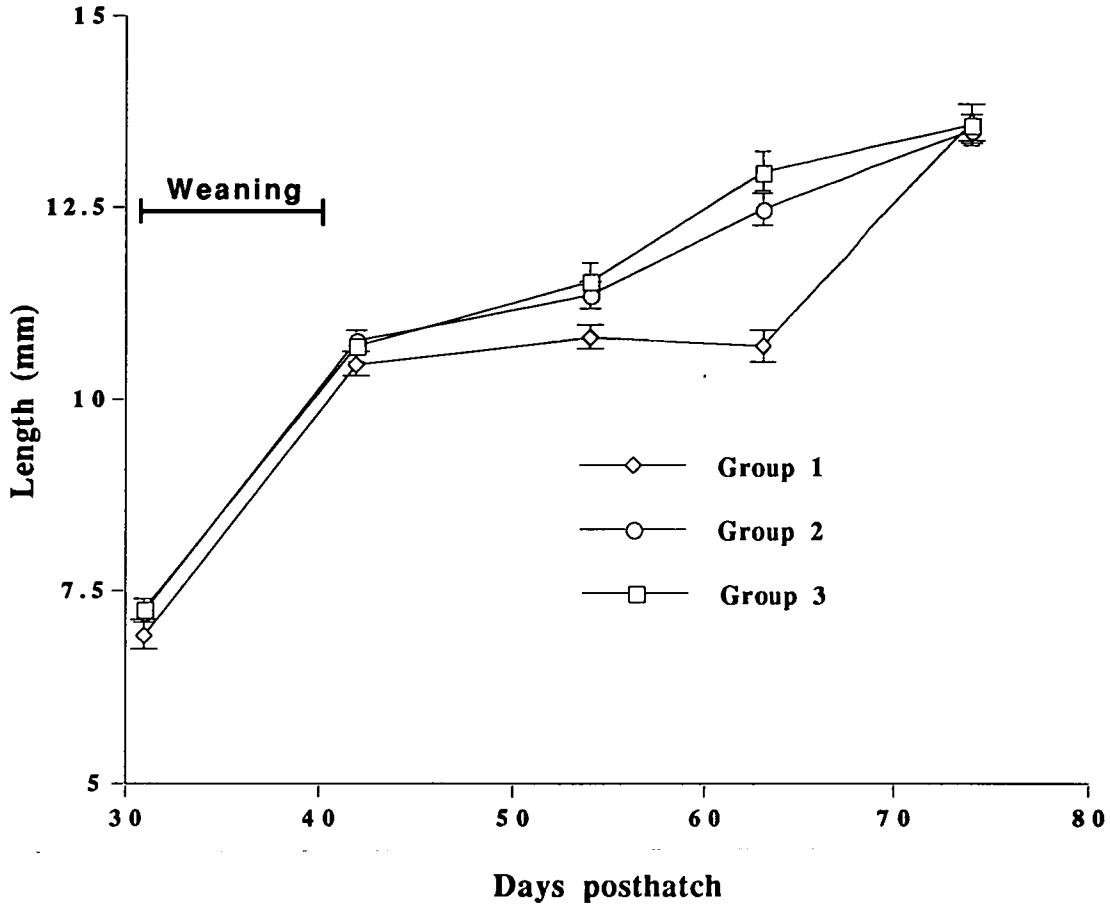


Fig. 6.3.17. Growth in length ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned after feeding with live prey enriched with Frippak (Group 1), Nutri-Pack (Group 2) or Microalgae followed by Frippak after day 15 post-hatch (Group 3).

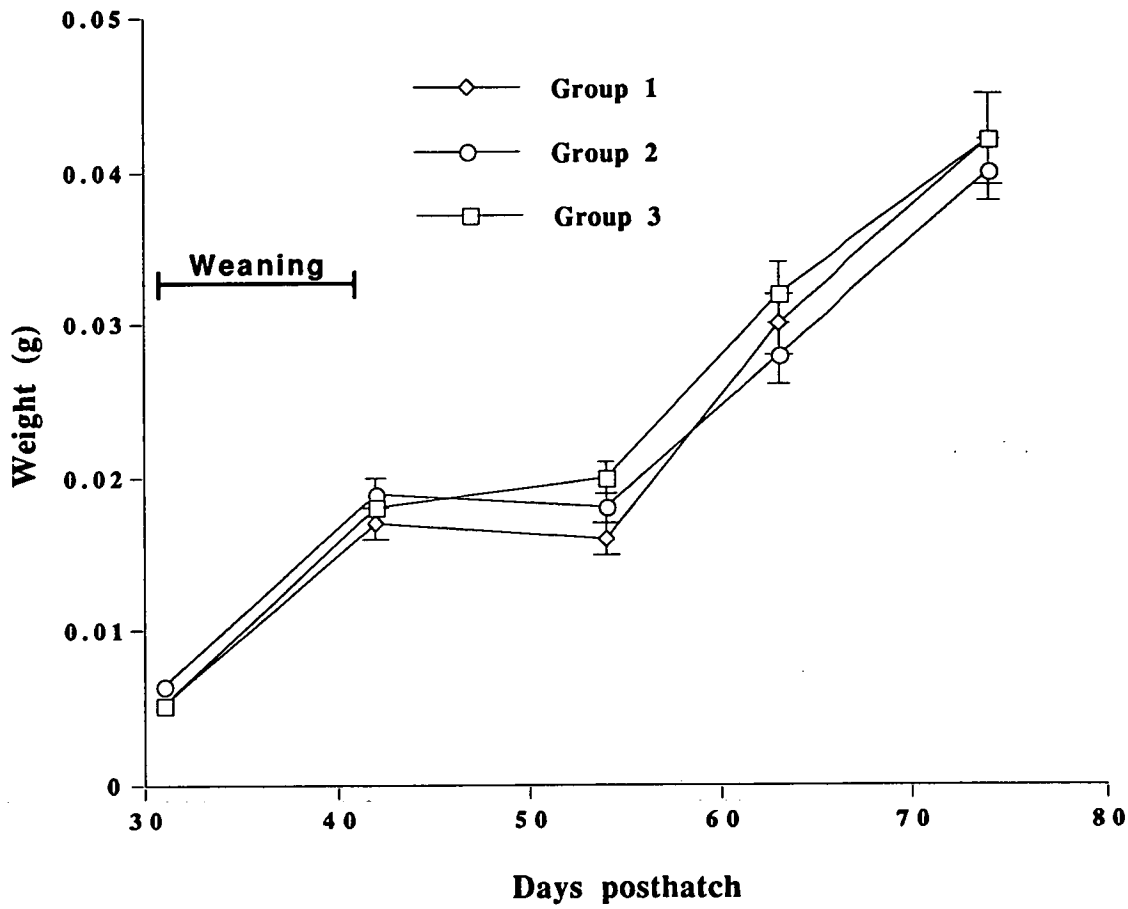


Fig. 6.3.18. Growth in weight ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned after feeding with live prey enriched with Frippak (Group 1), Nutri-Pack (Group 2) or Microalgae followed by Frippak after day 15 post-hatch (Group 3).

Points with no visible error bars have very small s.e.

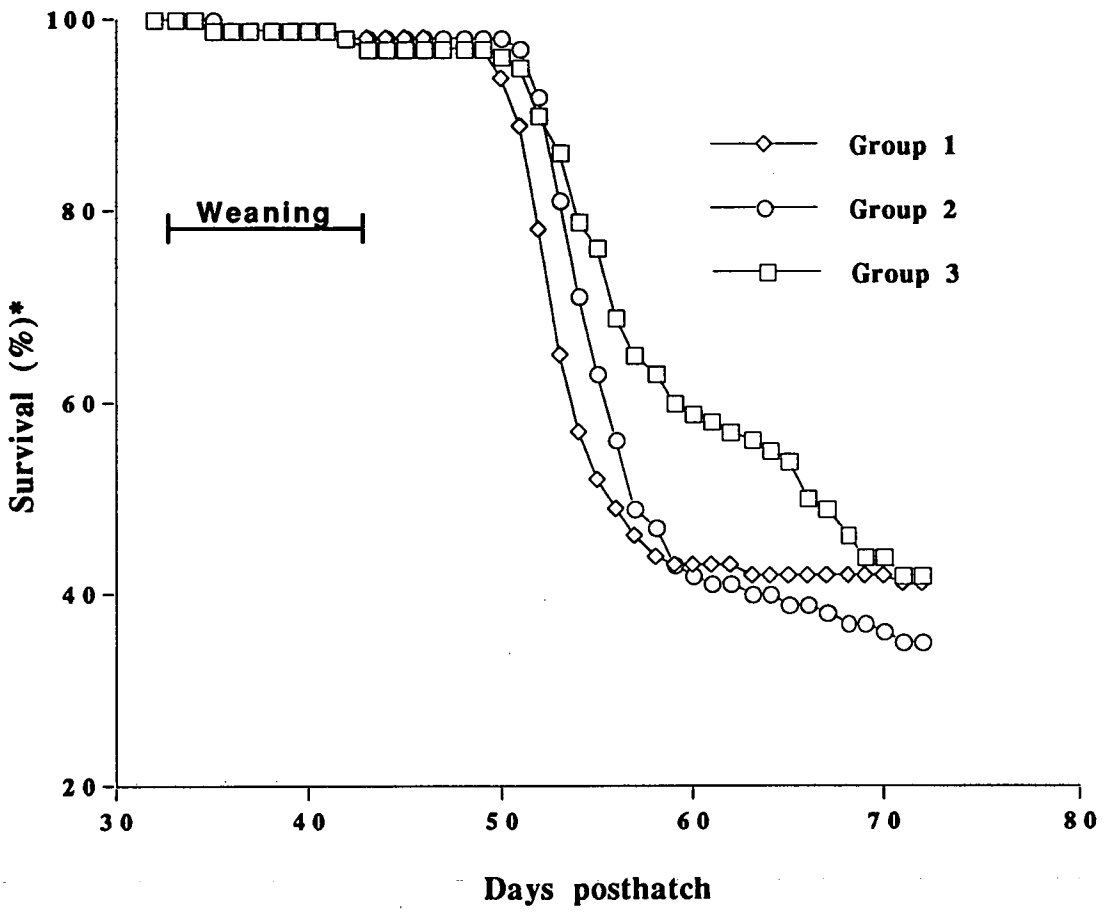


Fig. 6.3.19. Mean survival rates of *Rhombosolea tapirina* weaned after feeding with live prey enriched with Frippak (Group 1), Nutri-Pack (Group 2) or Microalgae followed by Frippak after day 15 post-hatch (Group 3).

* Survival is observed survival. Actual survival is given in Table 6.3.12

Maximum s.e. = ±8.82% (Frippak), ±16.92% (Nutri-Pak) and ±7.81% (algae/Frippak) over the experimental period.

### (ii) Feed rate prior to weaning

Feeding different rates of *Artemia* prior to weaning, resulted in no significant differences (P>0.05) between treatments in terms of growth or survival rates (Tables 6.3.14. and 6.3.15.). A decrease in length of the fish in group 1 was recorded in the first 10 days and was probably due to reduction in the length of the caudal fin (Figs. 6.3.20. and 6.3.21.). Many fish in these tanks were observed biting the fins of other fish, particularly those of the smaller ones, as *Artemia* became limiting. The growth rates, particularly in the case of weight, improved considerably in groups 1 and 2, as soon as weaning commenced and artificial food was introduced.

There were no significant differences (P>0.05) in survival between treatments (Fig. 6.3.22.). Mortalities in group 1 fish occurred at a relatively constant rate commencing at the beginning of the weaning period. The same was true of group 2 fish except that there was a marked increase in the mortality rate commencing at the end of the weaning period. The fish in group 3 showed the normal pattern with the onset of mortality occurring at the end of the weaning period and lasting for approximately 8-10 days.

**Table 6.3.14. Final lengths, weights and survival of *R. tapirina* juveniles weaned after periods of feeding with different feed rates of *Artemia* ($\bar{x} \pm$ s.e., n=3 replicates).**

| Live feed enrichment. | Final length (mm) | Final weight (g) | Survival (%) |
|---|---|---|---|
| Group 1. 1 *Artemia* /ml | 18.8 (0.7) | 0.12 (0.01) | 50.2 (3.9) |
| Group 2. 5 *Artemia* /ml | 21.2 (0.9) | 0.18 (0.03) | 41.8 (1.7) |
| Group 3. 10 *Artemia* /ml | 23.5 (1.7) | 0.23 (0.05) | 39.7 (2.2) |

There were no significant differences (P>0.05) between treatments.

**Table 6.3.15. Results of one-way ANOVA comparing the final lengths, weights and survival (arc sine √ transformed data) of *R. tapirina* weaned after feeding with live feeds enriched with different rates of *Artemia*.**

| Source | DF | SS | MS | F Ratio | P Value |
|---|---|---|---|---|---|
| Final length | | | | | |
| Model | 2 | 33.357 | 16.678 | 4.031 | **P>0.05** |
| Error | 6 | 24.827 | 4.138 | | |
| Total | 8 | 58.184 | | | |
| Final weight | | | | | |
| Model | 2 | 0.0196 | 0.0098 | 2.628 | **P>0.05** |
| Error | 6 | 0.0224 | 0.0037 | | |
| Total | 8 | 0.0420 | | | |
| Survival | | | | | |
| Model | 2 | 0.0188 | 0.0094 | 4.082 | **P>0.05** |
| Error | 6 | 0.0138 | 0.0023 | | |
| Total | 8 | 0.0327 | | | |

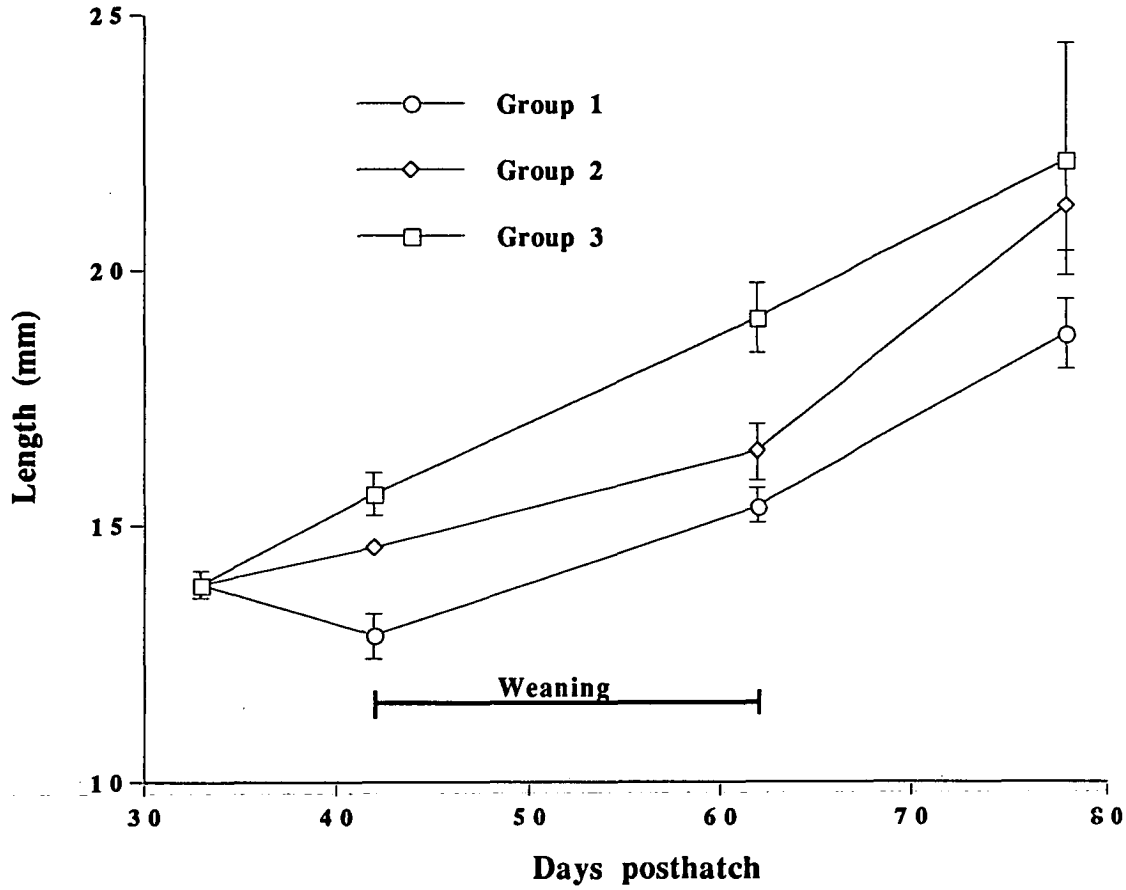


Fig. 6.3.20. Growth in length ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned after feeding with rates of either 1 *Artemia*/ml (Group 1), 5 *Artemia*/ml (Group 2) or 10 *Artemia*/ml (Group 3) for 10 days prior to weaning.

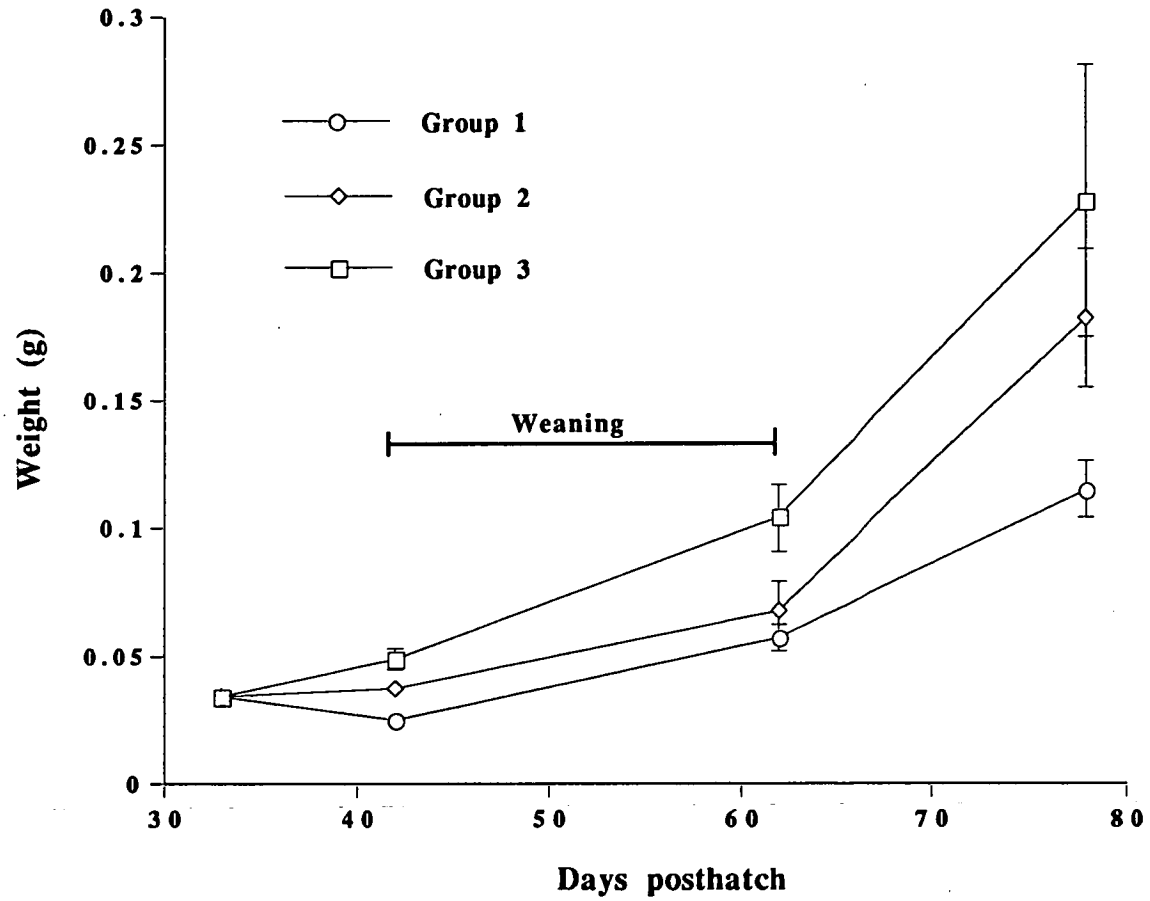


Fig. 6.3.21. Growth in weight ($\bar{x} \pm$ s.e.) of *Rhombosolea tapirina* weaned after feeding with rates of either 1 *Artemia*/ml (Group 1), 5 *Artemia*/ml (Group 2) or 10 *Artemia*/ml (Group 3) for 10 days prior to weaning.

Points with no visible error bars have very small s.e.

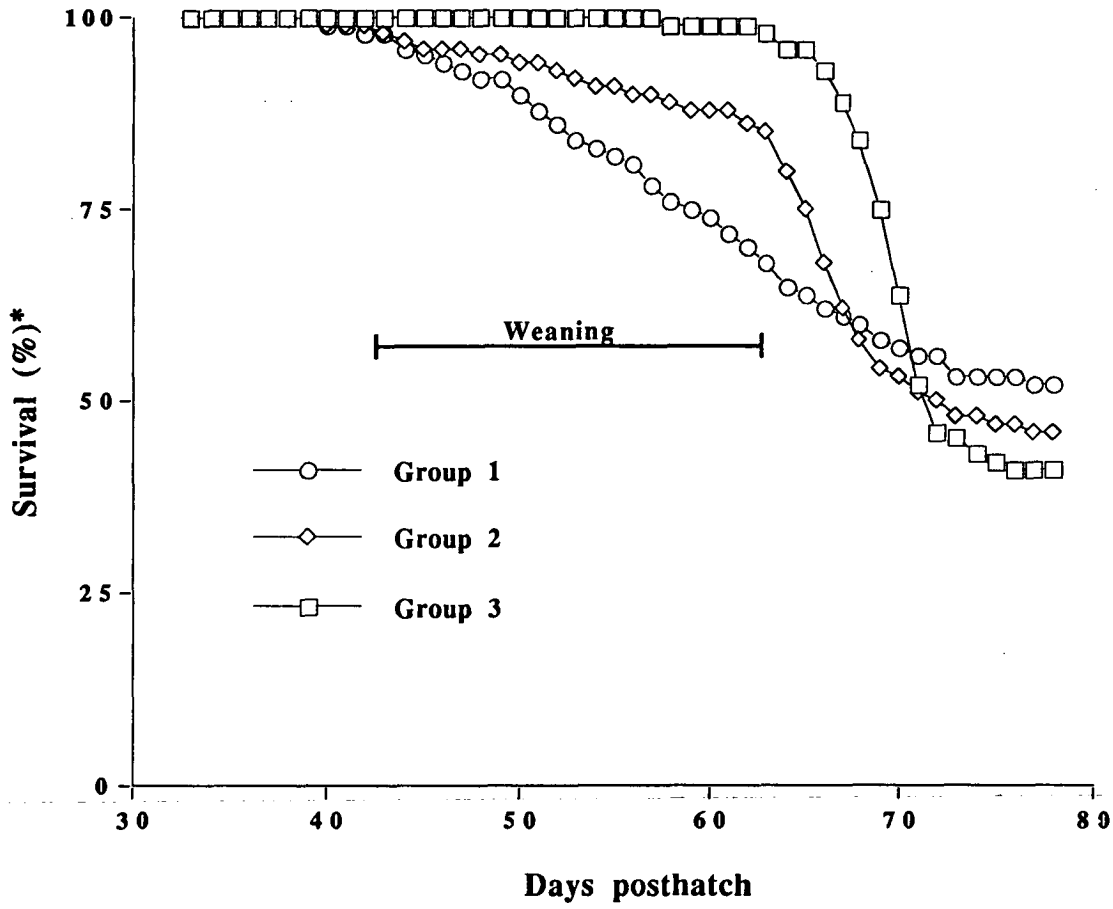


Fig. 6.3.22. Mean survival rates of *Rhombosolea tapirina* weaned after feeding with rates of either 1 *Artemia*/ml (Group 1), 5 *Artemia*/ml (Group 2) or 10 *Artemia*/ml (Group 3) for 10 days prior to weaning.

* Survival is observed survival. Actual survival is shown in Table 5.3.14.

Maximum s.e. = ±4.17% (group 1), ±6.63% (group 2) and ±4.98% (group 3) over the experimental period.

## 6.4. DISCUSSION

The results of the present study show that it is possible to wean the larvae of the greenback flounder (*Rhombosolea tapirina*) onto a dry diet with reasonable, though variable, success and after a period of feeding with enriched live diets (rotifers and *Artemia* sp.). Feeding with the live feeds only at night during the weaning period, as described by Gatesoupe (1983), was successful as a weaning method. Survival rates are comparable with those recorded for commercially produced marine species. Values in Table 6.4.1. compare the results of the present study with some of the published data.

**Table 6.4.1. The results of some weaning trials with temperate commercial marine fish.**

| Species | Age at weaning (d) | Length of changeover (d) | Survival (%) | Temp (°C) | Reference |
|---|---|---|---|---|---|
| *R. tapirina* | 23 | 10 | 82.2 | 15 | This study |
| *S. maximus* | 25 | 5 | 85 | 16-21 | Riaza & Hall, 1993 |
| *S. solea* | 10 | 0 | 70 | 18 | Applebaum, 1985 |
| " | First-feed | 0 | 20 | 15 | " |
| " | 10 | 5-6 | 44 | 18-20 | Gatesoupe, 1983 |
| *D. labrax* | 30-35 | 0 | 85 | 19 | Person-Le Ruyet *et al.*, 1993 |

*The effect of different weaning diets*

In the present study dry diets were used exclusively for weaning. The addition of fresh ingredients, or the use of fresh feeds was not considered a commercially viable option for improving the weaning success rate on a large scale. Dry diets are used successfully for the weaning of flatfish in European commercial facilities (Person-Le Ruyet, 1990). However, Métaillier *et al.* (1981b) obtained the highest growth rate of larval *S. solea* when using a dry pellet containing 45% squid offal, reclaimed filleting offal and Norway lobster offal. Frozen molluscs and a fish meal based dry pellet gave the worst results. The addition of a natural attractant such as fresh, chopped, molluscs or polychaetes considerably improved the growth rate. Gatesoupe (1983) found a moist pellet to be most successful for weaning *S. solea*.

The artificial feed composition did not appear to have a major effect on weaning success during this study. However, the fish were weaned very late with small particle sized diets which possibly accounts for the poor overall survival rates, compared with later experiments. The Skretting diet with a slightly larger particle size gave the best

survival rate while Sevbar, a diet designed specifically for marine fish and having a lower fat content, gave slightly better growth, but reduced survival. Wankowski and Thorpe (1979) showed that the particle size of salmon diets is critical in obtaining maximum growth, with only one particle size giving optimal growth rates for each size of salmon investigated. Larger or smaller particles than the optimum, resulted in reduced growth. Future studies with *R. tapirina* should be directed towards matching the particle size of the diets used for weaning, with the gape size of the larvae and identifying the nutritional requirements of the larvae.

*The effect of different stocking densities*

The stocking density trial was conducted to establish the importance of stocking density as a possible factor influencing growth and survival in weaning experiments. The high mortality observed during weaning trials, corresponding to a sudden alteration in the stocking densities of some tanks, could possibly influence the interpretation of other weaning experiments, if significant. However, over the range of stocking densities investigated (5-20 fish/l), the results of this trial showed that stocking density is not a factor requiring consideration. Devresse *et al.* (1991) recommend a stocking density of 10 fish/l for weaning. Gatesoupe and Luquet (1981/82) tested stocking densities of 40 and 130 larval *S. solea*/l in order to determine whether a higher stocking density would reduce the over distribution of artificial food, and consequent reduction in the water quality, in rearing tanks, but no advantage was obtained by using a high stocking density.

*The duration of the weaning period*

The duration of the weaning period had no effect on the survival rate of *R. tapirina.* However, a 5 day period resulted in reduced growth rates and was, therefore considered to be unacceptably short. Increasing the weaning period to 10 or 20 days improved the growth rate significantly. Extending the weaning period beyond 10 days increased the cost of *Artemia* while delaying the onset of mortality, without improving the survival rate. The same phenomenon has been observed with *S. solea* (Bromley and Sykes, 1985). Gatesoupe and Luquet (1981/1982) obtained better growth rates of *S. solea* by using an abrupt change from live *Artemia* nauplii to artificial food, but this was associated with low survival of only 15-28%. Crawford (1984a) recorded 0% survival (100% mortality) when attempting this method with *R. tapirina.*

In addition to increased costs of production associated with extended weaning periods is an apparent vitamin C deficiency observed as foreshortened operculi and spinal deformities (PLATE 21). The cause of the deficiency may be behavioural; the larvae

tending to ignore the artificial feeds until exhaustive searches for livefeed have proven unsucessful. The result of this is an accumulation of food on the tank bottom, from where vitamin C and other nutrients are leached, until the fish reluctantly eat it. The weaned juveniles resulting from a 10 day weaning period were the first to show reduced symptoms of vitamin C deficiency. However, even these fish exhibited some signs of deformity. Therefore, the reason for vitamin C deficiency symptoms remains unclear and requires further research. Person-Le Ruyet (1990) mentions that turbot (*Scophthalmus maximus*) also suffer from vitamin C deficiency and therefore, this may be a problem with flatfish in general, either due to behavioural anomalies or higher nutritional requirements.

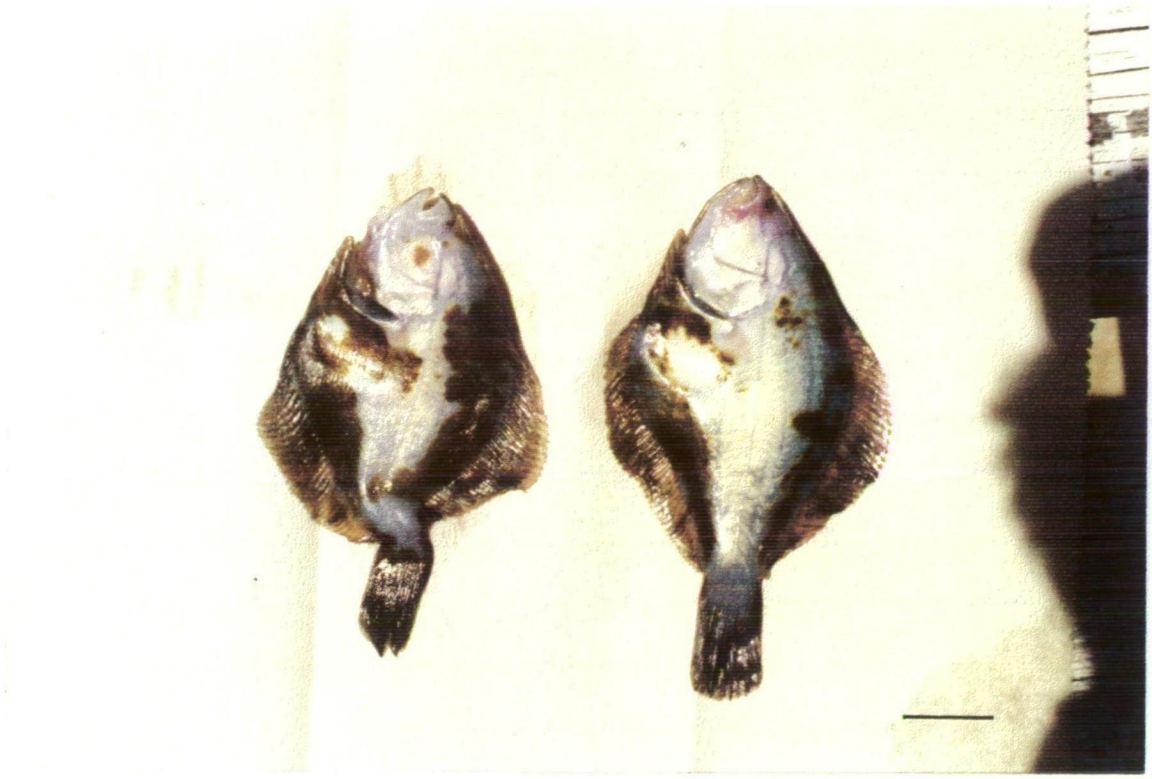

PLATE 21. The blind side of cultured *R. tapirina* juveniles, showing extensive dark pigmentation, foreshortening of the operculum and lordosis of the vertebral column. Scale bar represents 1 cm.

*The timing of weaning*

The larvae of *R. tapirina* can be successfully weaned onto a dry pellet before metamorphosis using a 10 day changeover period and the survival and growth rates are high. Gatesoupe and Luquet (1981/1982) were able to wean the larvae of *S. solea* onto a semi-moist diet before metamorphosis. The best survival rates were obtained by feeding frozen *Artemia* nauplii from day 11 to day 15 post-hatch. A survival rate of 40% was achieved to 50 day post-hatch. Bromley (1978) achieved survival rates of 10-70% when weaning larval *S. maximus* of 6-17 mm. The smaller the larvae the more

difficult it was to wean them, but additions of live food during weaning resulted in higher survival rates. Bromley and Howell (1983) carried out weaning trials with *S. maximus* at days 23-37 post-hatch. The results were highly variable with survival rates of 45-95% recorded.

In the present study with *R. tapirina* it was shown that if weaning is initiated on day 23 post-hatch with larvae of good quality, it is possible to achieve a survival rate of 80% without greatly affecting the growth rate. This strategy results in a considerable saving in *Artemia* and consequently, a lower cost of production. Person-Le Ruyet *et al.* (1993) have stated that the live prey biomass used for the production of *D. labrax* would be reduced by 60% if weaning was carried out at day 25 rather than on day 35 post-hatch. Gatesoupe and Luquet (1981/1982) recorded a significant reduction in the growth rate when weaning *S. solea* to a moist pellet before metamorphosis, but this was offset by a significant reduction in the number of nauplii required per metamorphosed juvenile, as compared to weaning after metamorphosis. A final mean weight of 27 mg was achieved at day 70, for a consumption of 2,800 *Artemia* nauplii, compared to 3,400 nauplii used by Fuchs (1979) for post metamorphosis weaning. An abrupt changeover from live to artificial feeds yielded considerable savings in the number of nauplii required per metamorphosed sole (50-90 mg final mean weight for a consumption of 1,980-2,660 *Artemia* nauplii). Gatesoupe (1983) obtained mean weights of 189-414 mg on day 70 with an *Artemia* consumption of 3,000 nauplii per metamorphosed sole, when attempting weaning before metamorphosis.

Juvenile *R. tapirina* over 50 days old were more difficult to wean than the younger fish possibly due to the physiological adaptations of the digestive system, over a longer period, to suit the live diets. Applebaum (1985) showed that the longer the larvae of *S. solea* were fed on *Artemia*, the more difficult it was to wean them onto the prepared diet. This may be due to the lack of specialisation of the digestive enzymes in the gut at an early stage. It has been shown that the feed consumed by larval fish affects the digestive capacity and enzyme composition of the gut (Hofer, 1985; Segner *et al*, 1993). The stomach of *R. tapirina* is fully differentiated by day 20 at 15°C (section 5.5.6.) and probably begins to produce gastric secretions which have not become specialised in their function and can be adapted to artificial diets. Segner *et al.* (1993) give evidence to suggest that the type of feed given to larval fish can affect their digestive physiology indefinitely. These authors also showed that the major difference between larval and juvenile fish is the lack of a functional stomach and this may be the reason for the inability of early larvae to digest artificial diets, rather than the lack of digestive enzymes.

Two alternative theories are that the pelagic behaviour of the larvae prior to metamorphosis may be beneficial in adapting them to feed on dry feed particles in the water column (Applebaum, 1985) or that the larvae may become conditioned to feeding on moving particles and therefore, larvae that have been fed with moving prey for long periods, become conditioned to this type of food. Barnabé and Guissi (1994) showed that using rotifers immobilised by cooling, in conjunction with microparticles, from first-feeding, improved growth and survival of *D. labrax* larvae and allowed weaning to take place successfully at day 20 post-hatch. The reason for this appears to be that the larvae never become conditioned to feeding on moving prey.

*The effect of live feed quality*

In the present study with *R. tapirina* the quality of the live feeds offered to the larvae and the quality of the larvae themselves, were considered to be the most important factors affecting weaning success as measured by the survival and growth rates. Larvae fed with live feeds enriched with commercial enrichment diets, grew faster and weaned more successfully than those fed on microalgae-enriched prey. Person-Le Ruyet *et al.* (1993) found that the survival rate of sea bass (*Dicentrarchus labrax*) larvae weaned from day 20 was highly dependant on the quality of the larvae, measured by the larval growth rate. Bromley and Howell (1983) considered that weaning success may be dependant on the quality of the live foods used before weaning begins. Larvae of *S. maximus* fed on *Artemia* metanauplii, were weaned more successfully than larvae fed on nauplii of San Francisco Bay *Artemia.* If the larvae of *D. labrax* are weaned after feeding with DHA deficient live feeds, the result is poor survival and growth, even when weaned using a previously successful weaning regime (Person-Le Ruyet, 1993).

In Trial 2 section 6.2.6. (i) of the present study on the affect of live feed quality on weaning, there were no differences between treatments, but the growth of the larvae prior to weaning in this trial was also very poor, compared to that achieved in previous years. The poor quality of the larvae in this case was probably due to small eggs of poor quality, and poor quality larvae at the commencement of exogenous feeding. The high stocking densities through metamorphosis, also resulted in food limitation and partial starvation during the important developmental processes occurring at this stage. The affect is still the same as that observed in Trial 1: poor growth and survival due to poor larval quality rather than any affect of the weaning process itself.

The use of algae as an enrichment for rotifers and *Artemia* during the first 15 days of larval rearing had a profound effect on the growth and survival rates of juvenile *R.*

*tapirina*, which did not become apparent until after metamorphosis and weaning to an artificial diet. This may help to explain the improvement in larval survival and quality that is often claimed when using algae in the larval rearing tanks (the 'green water technique'). A number of advantages are claimed for the 'green water technique' for larval rearing: water purification (Jones, 1970), continuous enrichment of live feeds (Jones, 1972, Scott and Middleton, 1979), light diffusion (Naas *et al.*, 1991) and enzyme stimulation due to early ingestion of algal cells (Naas *et al.*, 1992). None of these theories has yet been substantially proven (Tamaru *et al.*, 1994).

It may be simply that an unknown nutritional component of the algae is required to produce larvae in optimum condition, and that this is obtained through the live feeds first ingesting the algal cells. If this is the case then the 'clear water' technique for larval rearing may prove to be just as successful in terms of larval condition so long as algae is used in conjunction with high HUFA commercial diets for enrichment during the first stages of larval rearing.

An alternative explanation is that the bacterial populations in the enrichment tanks can affect the bacterial species composition on the surface of the rotifers and this in turn, affects the bacterial population of the larval fish gut. The bacterial species composition of algal fed rotifers has been shown to be more varied, with lower total numbers of species, but greater species diversity, than those of emulsion or commercial enrichment-fed rotifers. The latter are typified by low species diversity and high total numbers of opportunistic bacteria such as *Vibrio* sp. This difference in the bacterial population structure of the rotifers is reflected in the bacterial populations of the rearing tank water and of the primitive early larval gut (Blanch *et al.*, 1991).

The same effect is produced in larval rearing tank water when using the 'green water' technique of culture. The bacterial species composition is typified by slow growing species in 'green water' and fast growing opportunistic species in 'clear water' systems (Skjermo and Vadstein, 1993). Skjermo and Vadstein (1993) showed that halibut (*Hippoglossus hippoglossus*) larvae contained three times as many bacteria in the gut if cultured in 'clear water' compared to those cultured in 'green water', even though the bacteria density in the water was lower. The bacterial species composition of the gut was not closely related to that of the rearing water, but was different in the 'green water' tanks from that in the 'clear water' tanks and may have reflected the bacterial species composition on the surfaces of the prey animals, although this was not determined.The importance of the bacterial flora of the larval fishes' gut on digestion of food is, as yet, unknown, but may be an important component of the larval fishes digestive capacity.

The significant improvement in post weaning growth, observed in the present study, in fish that had been previously fed for the early larval period with algae enriched prey, may therefore, be attributed to greater digestive capacity afforded by the composition of the bacterial microflora of the larval gut. Munro and Birkbeck (1993) showed that the gut composition of *S. maximus* larvae is closely related to that of the rotifers. Munro *et al.* (1993) showed that the majority of bacteria associated with rotifers were located on the external body surface and must therefore be present in the rearing water. In 'green water' cultures it is therefore probable that the bacteria in the water are passed on to the fish gut, via the rotifers and this may be the major benefit of 'green water' culture techniques. The use of algae as an enrichment or in the 'green water' larval rearing technique, may therefore have direct benefit in improving the bacterial composition of the larval gut and increasing the efficiency of digestion.

Reduction of the total bacterial load in rotifer cultures, using lactic acid bacteria, has also been shown to result in improved growth rates of *S. maximus* larvae (Gatesoupe, 1990, 1991). In this case the main bacteria in the cultures were *Vibrio* sp. and *Aeromonas* sp., both opportunistic species. A reduction in these bacteria would have the same effect as described above. An improvement in growth was not observed in *Artemia* fed larvae, possibly due to rinsing in freshwater, which would have killed any bacteria on the body surfaces (Gatesoupe, 1991).

*The effect of partial starvation*

A reduction in condition caused by partial starvation after the completion of metamorphosis and immediately prior to weaning, had little affect on weaning survival. The results of this trial therefore, lead to the conclusion that low feed rate immediately prior to weaning simply reduce the growth rate rather than affecting survival. In a commercial situation it is undesirable to reduce the growth rate and therefore the feed rate prior to weaning should be optimised.

The main findings of the present study were that:

1. Weaning of *R. tapirina* onto a conventional dry pelleted diet is not affected by the artificial diet used, provided it is a complete and well-balanced diet, such as those used in this study and provided that the size of the particles is matched to the gape size of fish.

2. Weaning of *R. tapirina* onto a conventional dry pelleted diet is unaffected by stocking densities of between 5 and 20 fish /l.

3. Weaning of *R. tapirina* onto a conventional dry pelleted diet can be achieved effectively with a 10 or 20 day overlap during which the live feed is reduced and the artificial diet increased.

4. Weaning of *R. tapirina* onto a conventional dry pelleted diet can be achieved most effectively if carried out between days 23 and 50 post-hatch; a survival rate of 83% being achievable.

5. Weaning of *R. tapirina* onto a conventional dry pelleted diet is most successful if the larvae are fed with live prey of optimal quality from first-feeding thus maximising growth rates.

6. Weaning of *R. tapirina* onto a conventional dry pelleted diet can be improved by using algae-enriched live feeds for the larvae until day 15 post-hatch.

7. Weaning of *R. tapirina* onto a conventional dry pelleted diet is not improved by starving the larvae prior to weaning; this results in a reduction of growth.

# CHAPTER 7

# POST-WEANING GROWTH

## 7.1. INTRODUCTION

There are a number of factors which affect the growth rates of cultured fish. These are: 1. the nutritional composition of the diet and the feeding strategy, 2. the water flow rates and design of the holding tanks, 3. the water quality including temperature, salinity and oxygen content, and 4. the condition of the fish in terms of stress, disease and the degree of maturation. Most of the literature on post-metamorphosis growth in cultured flatfish involves the turbot (*Scophthalmus maximus*), as this is still the only species on which intensive trials have been carried out.

Guillaume *et al.* (1991) reviewed the literature on the nutrition of flatfish and found a very high protein requirement of around 60%, irrespective of the lipid level. It also appears that protein may be used for energy before lipid, as lipid is excreted at high protein levels. Caceres-Martinez *et al.* (1984) showed that at protein levels of 37.5% the growth rate improved with increased dietary energy, but at higher protein levels of up to 69.8% the growth rate decreased with dietary energy. Sea bass (*Dicentrarchus labrax*) showed the best growth and food conversion rate (FCR) with diets containing high protein levels of up to 60%. However, lower protein levels resulted in an improvement in the protein efficiency ratio (PER = g live weight gain/g protein eaten). Substituting carbohydrates for lipids at a given protein level was found to result in improved growth, FCR and PER (Métaillier *et al.*, 1981a).

Using diets containing 3,000 kcal/kg, Adron *et al.* (1976) found that a reduction in protein content from 50 to 35% caused no reduction in the growth rate of *S. maximus* and was accompanied by an improvement in the PER. Dietary carbohydrate had a similar effect, but much less marked. The growth rate of plaice (*Pleuronectes platessa*) improved with carbohydrate levels of up to 10% in the diet (Guillaume *et al.*, 1991). Interestingly, Adron *et al.* (1976) found that the fat content of the fish remained lower than that of wild fish, even after feeding a diet containing 50% non-protein calories for 18 weeks.

It has been shown that *S. maximus* and *P. platessa* do not have the necessary microsomal desaturases to desaturate fatty acids and therefore, it is necessary to supply HUFA's of the n-3 series in the diet (Owen *et al.*, 1972; Cowey *et al.*, 1976). Owen *et al.* (1975) and Léger *et al.* (1979) showed that there was some elongation of 18:3n-3 to n-3 HUFA in *S. maximus*, but not as much as that shown by rainbow trout (*Oncorhynchus mykiss*).

Although cultured *S. maximus* are still fed mainly with moist diets and trash fish it has been shown that dry diets are accepted. Bromley (1980) showed that there was no

effect on the growth rate with diets containing from 0-74% moisture. The feeding rate was found to exert the major influence on the growth rate with a ration of 6% biomass/d (dry weight) resulting in the highest growth rates. A FCR of 0.63:1 was achieved. Person-Le Ruyet (1990) states that in commercial culture it is normal to achieve FCRs of around 1:1 with a feed rate of 1% biomass/d given in 1 meal.

Cripps and Poxton (1992) reviewed the literature on tank and cage design for ongrowing flatfish and concluded that circular tanks with a central outlet were optimal. The tanks should have a sloping floor, multiple inlets to promote even water flows and be larger than 1,000 l in capacity. Some form of stacking would allow more efficient use of floor space and this would be facilitated by using shallow tanks containing less water which would be lighter in weight.

Purdom *et al.* (1972) states that the water flow required for *S. maximus* at 18°C is 13.6 l/d/kg of fish, which is considerably lower than that required by salmonids (Speece, 1973). Brown *et al.* (1984) calculated the oxygen requirements of *S. maximus* and found them to be approximately two-thirds those of trout. A 100 g fish at 12°C uses 105 mg/kg/hour compared to 145 mg/kg/hour reported by Liao (1971) for rainbow trout (*Oncorhynchus mykiss*). Stocking densities of 25 kg/m$^2$ can be achieved with *S. maximus* juveniles of approximately 100 g, in a water depth of only 20 cm (Person-Le Ruyet, 1990).

Early maturity of cultured fish can lead to reduced growth rates. It appears that cultured fish reach a critical minimum size for maturation much sooner than wild fish due to increased growth rates under culture conditions (Polikansky, 1983; Thorpe, 1986). This phenomenon has been described for *G. morhua* (Atlantic cod) (Ousthuizen and Daan, 1974), *P. platessa* (Rijnsdorp, 1993) and *Sciaenops ocellatus* (red drum) (Thomas and Arnold, 1993). Thorpe *et al.* (1982) suggested that bimodality in juvenile Atlantic salmon (*Salmo salar*) was caused by early maturation of males leading to a reduction in the growth rate. However, this was contradicted by Villarreal and Thorpe (1985) who discovered that there was no differential allocation of resources to somatic or gonadal growth in either the large mode or the small mode. *S. salar* frequently mature as juveniles if the growth rate is too fast (Thorpe, 1986). Polikansky (1983) concluded that under stable conditions with abundant food supply, fish will grow rapidly and mature as soon as they are developmentally able to do so. Thorpe (1986) hypothesised that the trigger for maturation was the rate of acquisition of surplus energy or stored fat. Male *S. salar* have been shown to use mesenteric fat for maturation of the gonads and maturation is suppressed if fat levels are too low prior to potential maturation periods (Rowe *et al.*, 1991).

The specific objectives of the present study were:

1. To make a preliminary assessment of the performance of cultured *R. tapirina* under artificial conditions, using dry artificial diets.

2. To make a preliminary assessment of the percentage maturation in cultured *R. tapirina* during their first year.

## 7.2. MATERIALS AND METHODS

### 7.2.1. Performance on a dry diet

This experiment was not intended to be a definitive study but was designed to give some indication of the performance of *R. tapirina* juveniles under intensive culture conditions, and provide some base line data for comparison with future studies. For this reason and due to the limited availability of tank space, the two feed rates were used at different times and cannot therefore be directly compared. It would have been better to have used a range of feed rates with three replicates of each, all compared over the same time period. However, this was not possible with the available equipment.

A set of three circular, flat-bottomed, brown fibreglass tanks of 300 l volume in a recirculating system, were each stocked with 1,000 flounder of 122 days post-hatch, graded for large size (initial length=33.60 ± 0.577 mm, weight=63.6 ± 0.043 g; $\bar{x}$ ± s.e.). A fourth tank of the same design in the same system was stocked with 2,500 flounder of the same age, but smaller size (length=25 ± 0.678 mm, weight=0.272 ± 0.017 g; $\bar{x}$ ± s.e.), selected by grading through a mesh net. Tanks were undercover, but exposed to natural daylength and temperature. The trial was started after an acclimatisation period of 13 days. Daylength at this time ranged from 14-15 hours over the 28 day experiment. Temperature fluctuated daily from 10-18°C at the start of the experiment, up to 12-19°C at the end. Flow rates were 10 l/min, with water entering at the surface to give a circular flow and leaving via a centrally placed bottom drain, covered with a mesh screen. Feeding was carried out by hand at least eight times daily with an artificial dry salmon diet manufactured by Skretting (0.6G and 1.0G, proximate composition = 51.7% protein; 13.9% fat; 17.3% carbohydrate and 50.9% protein; 18.4% fat; 15.9% carbohydrate, respectively). Feed rate was 10% of total biomass per day, over the first 14 day period, reduced to 5% for the second 14 day period. Sampling of 50 fish from each tank was carried out at the start of the trial, after 14 days and after 28 days. At each sampling date fish were randomly selected and anaesthetised in a 40 ppm solution of benzocaine before measuring and weighing.

Specific growth rate (S.G.R.) was calculated using the formula:

$$S.G.R. = \frac{\ln(W_t) - \ln(W_o)}{t} \times 100$$

Food conversion rate (F.C.R.) was calculated using the formula:

$$F.C.R. = \frac{\text{Dry weight of food fed}}{\text{Increase in wet weight of fish}}$$

Condition factor (K) was calculated using the formula:

$$K = \frac{\text{weight}}{\text{length}^3}$$

### 7.2.2. Early maturation

#### i) Bridport

A batch of 2,000, 8 month-old flounder, was transferred from the hatchery in February 1992 to the growout site at Bridport, (initial length= 56.3 ± 1.5 mm, weight=3.0 ± 0.3 g; $\bar{x} \pm$ s.e.). These fish were fed *ad libitum* with steam-pressed trout pellets (proximate composition = 38.4% protein; 16% fat; 2.7% fibre). The fish were held in a circular galvanised steel tank with a concrete base and a central outlet. Water was pumped direct from the estuary of the Brid River with no filtration or temperature control. A shade-net was placed over the tank, as protection from direct sunlight, but otherwise the fish were subjected to the natural photoperiod. In September 1992 (after 7 months in the experiment) a random sample of 100 fish was weighed, measured and the sex determined by visual observation of the gonad in females or by the release of sperm under gentle pressure, in males.

#### ii) Launceston

The remaining 400 fish were cultured in two brown circular fibreglass 300 l tanks in a recirculation system at the University of Tasmania in Launceston. The system was housed in a room with natural photoperiod and no temperature control. Feeding was *ad libitum* using the same type of feed as that used at Bridport. In September 1992 a random sample of 100 fish was examined in the same manner as those at Bridport.

The degree of lipid deposition in the liver was assessed histologically, at the Fish Health Unit, Mount Pleasant Laboratories, Tasmania.

## 7.3. RESULTS

### 7.3.1. Performance on a dry diet

All fish showed significant growth over the trial period, although the smaller fish in tank 4 yielded considerably poorer performance (Tables 7.3.1. and 7.3.2.). Tanks 1, 2 and 3 showed very similar trends over all assessment criteria used.

**Table 7.3.1. Weights and lengths for juvenile *R. tapirina* fed over 28 days on a dry salmon diet ($\bar{x} \pm$ s.e., n=3 replicates for the large and n=1 for the small fish).**

| Fish size | Initial weight (g) | Initial length (mm) | Weight at 14 days (g) | Length at 14 days (mm) | Final weight (g) | Final length (mm) |
|---|---|---|---|---|---|---|
| Large | 63.6 (0.04) | 33.6 (0.6) | 1.17 (0.08) | 40.7 (1.3) | 1.95 (0.17) | 48.8 (1.7) |
| Small | 0.272 (0.02) | 25.0 (0.7) | 0.37 (0.03) | 27.9 (0.8) | 0.50 (0.04) | 31.4 (1.0) |

**Table 7.3.2. Specific growth rate (S.G.R.), food conversion rate (F.C.R.) and condition factor (K) of juvenile *R. tapirina* fed for 28 days with a dry salmon diet at a feed rate of 10% b.w. for the first 14 days and 5% b.w. for the final 14 days ($\bar{x} \pm$ s.e., n=3 replicates for the large and n=1 for the small fish).**

| Parameter | 1st. 14 days | | 2nd 14 days | |
|---|---|---|---|---|
| | Large | Small | Large | Small |
| S.G.R. (%/day) | 4.4 (0.1) | 2.2 | 3.6 (0.5) | 1.4 |
| F.C.R. (dry wt. fed: wet wt. gain) | 1.42:1 (0.1) | 3.64:1 | 1.03:1 (0.2) | 3.02:1 |
| K | 1.70 (0.02) | 1.7 | 1.71 (0.1) | 1.45 |

For tanks 1, 2 and 3, a 50% reduction in feed rate in the second period gave only a 17.2% reduction in S.G.R. with a 27.5% improvement in the F.C.R. and no affect on K. The final stocking density of the larger fish was approximately 6-7 kg/m$^3$.

Tank 4 showed a different pattern with a 50% reduction in feed rate resulting in a 35.8% decrease in S.G.R. and a 14.7% decrease in K for only a 17% improvement in F.C.R. All performance figures for this tank are well below those of tanks 1, 2 and 3.

### 7.3.2. Early maturation

Mean weights and lengths of flounder from the two sites are presented in Table 7.3.3. The means for total weight and length were similar at both sites. The mean lengths and weights of females at Bridport were greater than for any other groups. The main difference between males and immatures at the two sites was that immatures at Bridport were smaller than immatures at Launceston. Bridport males, Launceston males and Launceston immatures were similar in size. Many of the females produced eggs, but none were viable. The frequency distribution histograms for these data are shown in Fig. 7.3.1.

**Table 7.3.3. Weights and lengths for *R. tapirina* of different sexes cultured at two different sites.**

| LAUNCESTON | $\bar{x}$ | s.e. | Percent (%) | Minimum | Maximum |
|---|---|---|---|---|---|
| Male, Length (mm) | 136.3 | 2.56 | 32 | 105 | 167 |
| Imm., Length (mm) | 129.8 | 2.31 | 68 | 70 | 158 |
| Male, weight (g) | 43.1 | 2.64 | 32 | 17.4 | 74 |
| Imm., Weight (g) | 38.1 | 1.88 | 68 | 4.7 | 68.8 |
| Total, Length (mm) | 131.9 | 1.79 | 100 | 70 | 167 |
| Total, Weight (g) | 39.7 | 1.88 | 100 | 4.7 | 74 |
| **BRIDPORT** | | | | | |
| Female, Length (mm) | 151.8 | 2.32 | 16 | 135 | 173 |
| Male, Length (mm) | 133.2 | 2.26 | 38 | 110 | 165 |
| Imm., Length (mm) | 122.7 | 2.28 | 46 | 76 | 156 |
| Female, Weight (g) | 60.35 | 3.71 | 16 | 37.7 | 95.4 |
| Male, weight (g) | 35.9 | 2.03 | 38 | 19.7 | 70 |
| Imm., Weight (g) | 28.4 | 1.64 | 46 | 6 | 57.4 |
| Total, Length (mm) | 131.4 | 1.73 | 100 | 76 | 173 |
| Total, Weight (g) | 36.4 | 1.64 | 100 | 6 | 95.4 |

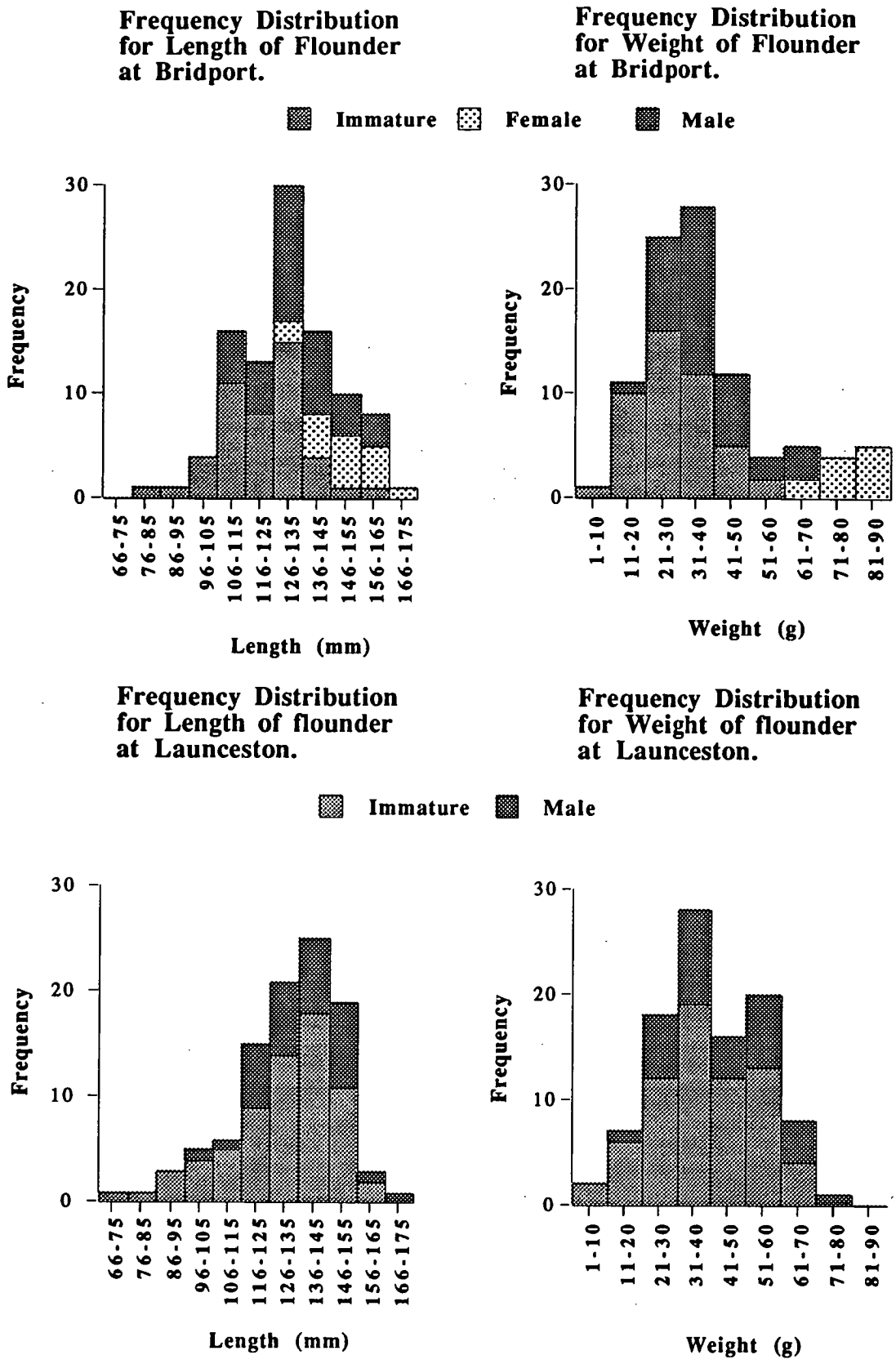


Fig. 7.3.1. Frequency distributions of *R. tapirina* cultured at Launceston and Bridport, showing the differences in frequency of males and females at the two sites.

## 7.4. DISCUSSION

*Performance on a dry diet*

In the present study the growth rates of cultured greenback flounder (*Rhombosolea tapirina*) juveniles, on a dry diet were high, and the food conversion rates were good for larger, graded fish. A feed rate of 5% appeared to give better food conversion rates than 10%, but the fish were older and this may have contributed to the lower feed requirement. Jackson (1988) found that Rainbow trout (*Oncorhynchus mykiss*) of 0.5-2 g require 5.1-5.5% dry feed per day, agreeing with the results for *R. tapirina* in the present study. Deniel (1976) carried out feeding trials with wild caught juvenile turbot (*Scophthalmus maximus*) using a moist, fish meal based artificial food, and found that the normal daily intake of dry food was 2% of the bodyweight. The growth rate accelerated after the fish had reached 20 g yielding an average weight of 62 g after 12 months. A comparison of growth rates on artificial food and natural food, consisting of molluscs and fish, was undertaken (Deniel, 1976). Artificial food yielded faster growth rates, over the first 9 months. However, the natural food gave better overall growth. Kuhlmann *et al.* (1981) obtained the highest growth rates by using a mixture of herring meat and mysids, closely followed by herring meat on its own. Kirk and Howell (1972) found that with plaice (*Pleuronectes platessa*) a diet of chopped enchytraeid worms (*Lumbricillus rivalis*) gave higher growth and lower food conversion than the artificial foods tested. The nutritional requirements of *R. tapirina* are not yet known and further research in this area is required before optimal growth rates can be achieved.

In the present study it was found that small stunted fish exhibited slower growth rates. These fish may not have fully recovered from the weaning process or may have been in generally poorer condition. In commercial operations it may be necessary to cull a certain proportion of the smaller fish, in order to achieve better overall growth rates.

Temperatures of approximately 15°C were used in the present study and the optimal temperature for larval growth was shown to be 18-20°C (Section 4.3.3.). With heated water of approximately 18°C a considerably faster growth rate would probably be possible with *R. tapirina*. A nursery phase in heated water may be beneficial in accelerating the growth rate and providing juveniles suitable for growout at an earlier stage. Temperatures of 18-20°C are optimal for the growth of *S. maximus*, but they can tolerate 25-30°C. In heated seawater of 18°C, fish can reach 350 g in one year but 175 g is more usual (Person-Le Ruyet *et al.*, 1981). The Japanese flounder (*Paralichthys olivaceus*) is cultured in water temperatures of 12-27°C (Watanabe and Nomura, 1990) and the optimal temperature is 19°C (R. Murashige, pers. comm).

Optimal temperatures for the growth of sea bass (*Dicentrarchus labrax*) are 22-24°C and that for sea bream (*Sparus auratus*) is 30°C (Barnabé, 1990).

*Early maturation*

At an age of 15 months, 54% (16% female and 38% male) of *R. tapirina* reached maturity at the Bridport site under natural temperature and light conditions. Lower numbers (32%) of mature fish were recorded at Launceston and none of these were females. The high fat salmonid diet caused a high degree of surplus fat storage (unpublished data from the Department of Primary Industry , Mount Pleasant Laboratories, Tasmania), particularly in the liver and this could have been the cue for maturation in the first year. Further research into the dietary energy requirements may help to reduce this phenomenon. Thorpe *et al.* (1990) showed that early maturation in *S. salar* could be reduced by restricting the food intake during the winter when fat stores are laid down ready for maturation. The use of triploid induction techniques may be an alternative method of reducing maturation. This technique has been used successfully for the production of triploid *P. platessa* and *P. olivaceus* (Japanese flounder) (Purdom, 1972; Tabata and Gorie, 1988).

The fact that mature females occurred only at Bridport may be due to a photoperiod effect. The fish at Launceston were cultured within a building and therefore the light would have reached low levels in the evening at an earlier time than at Bridport. This would effectively have reduced the daylength and possibly delayed the onset of maturity. Some fish were observed to mature at Launceston after these data were collected. Temperatures at Launceston showed a much greater daily range due to the small size of the system and the lack of temperature control. This may also have influenced the degree and timing of maturation.

The results of the present study show that:

1. Growth and FCRs of *R. tapirina* fed with dry diets, are equal to those obtained with other commercial species. However, the nutritional requirements of the species require further research, particularly with respect to lipid and carbohydrate levels.

2. Early maturation is a potential problem and further research will be required to determine the effect on future growth rates and condition. Sterilisation techniques or monosex production techniques may be required to obtain profitable growth rates.

# CHAPTER 8

# GENERAL DISCUSSION

The results of the present study show that intensive hatchery production of greenback flounder (*Rhombosolea tapirina*) is feasible, and potential exists for expansion to large-scale production of the species. The major finding of the study was that standard marine fish hatchery techniques can be used effectively for larval rearing of *R. tapirina*, with reasonable survival and growth to post-weaning. The hatchery production techniques developed will require little modification for transfer to larger scale operations.

## 8.1. ONGROWING METHODS

Most commercial ongrowing of flatfish is carried out in flat-bottomed concrete tanks. In Hawaii the tanks are 8 x 8 x 1 m which gives a surface area of 64 $m^2$, a volume of 64 $m^3$ and a capacity of around 500-600 kg of Japanese flounder (*Paralichthys olivaceus*) (Murashige, pers. comm.). It may be feasible to use cages for growout, but these have not been successful with turbot (*Scophthalmus maximus*) in Europe due to the effect of surface wave action on the cage base. However, cages have been used successfully with halibut (*Hippoglossus hippoglossus*) (Martinez Cordero *et al.*, 1994). The use of elasticised cage walls has been investigated but the results are unclear. Growout in cages would be less costly than in shore-based tanks. In a report by Smith (1976) the ongrowing of *S. maximus* in floating cages was investigated and considered feasible. Hull and Edwards (1976) carried out trials with small sea cages moored in a sheltered bay with good tidal flow. The cages were 1.8 x 1.8 x 1.2 m deep with 12.7 mm square, plastic coated, weldmesh, with a volume of 2.9 $m^3$. In Japan, *P. olivaceus* are cultured in cages of 5-10 $m^2$, as well as land-based tanks (Kuronuma and Fukusho, 1984; Watanabe and Nomura, 1990).

A nursery stage of 2-3 months is probably required before transferring stock to growout facilities. Smith (1976) found that 60 g was a suitable size for transfer of *S. maximus* to cages and better growth was obtained if the fish were transferred in the warmer months of the year. In Japan *P. olivaceus* are transferred to cages at a length of 15.2-15.9 mm (Kuronuma and Fukusho, 1984).

The nutritional requirements of flatfish are not well documented and further research is required to identify the optimal feed formulation. Gibsons trout feed can probably be used to feed *R. tapirina* juveniles until market size of 500 g is achieved, possibly after 2 years. Food conversion rates of around 1:1 were achieved during the nursery stage under laboratory conditions, but it is unlikely that this will be possible on a large scale. Optimal stocking densities for *R. tapirina* have not been evaluated. In Hawaii a stocking density of 10-12 kg/$m^2$ is used for *P. olivaceus* (Murashige, pers. comm.). However, in Europe *S. maximus* are stocked at densities of 25-50 kg/$m^2$ and densities

of up to 120 kg/m$^2$ have been documented (Howard, 1974; Hull and Edwards, 1976). Person-Le Ruyet *et al.* (1981) state that the stocking density in sea cages is 10 kg/m$^2$ or m$^3$, but that this is probably rather low. Hull and Edwards (1976) used a maximum stocking density of 41 kg/m$^3$, with *S. maximus* in cages, but this was not thought to be the maximum possible. Stocking densities of 73 kg/m$^3$ have been achieved with plaice (*Pleuronectes platessa*) in floating cages (Howard, 1974).

## 8.2. ECONOMICS

Very little information on the costs of production is presently available. It is however, possible to make direct comparisons to the existing commercial culture of marine species in Europe.

In Europe the market price of juvenile sea bass (*Dicentrarchus labrax*) is A$0.74 each and for sea bream (*Sparus auratus*) it is A$0.86 each. The production cost for sea bass is A$0.37 per fingerling (Stanley, 1992). In Australia, barramundi (*Lates calcarifer*) fingerlings cost A$0.60 each, when cultured intensively, but may be reduced to A$0.20 each with the introduction of extensive production techniques (Treadwell *et al.*, 1991). *R. tapirina* are probably most similar to *S. auratus* as far as the culture method and production costs are concerned, although extensive production is a potential option. A large-scale marine fish hatchery in France, producing 1,000,000 marine fish fingerlings per annum, was set up at a cost of A$500,000 (Stanley, 1992). The cost of a hatchery for *R. tapirina* on the same scale, is likely to be similar.

Growout costs for *R. tapirina* are likely to be similar to those of Atlantic salmon (*Salmo salar*) as the limited data available suggest that production time and food conversion rates are similar. However, there will be a considerable increase in the cost if land-based tanks have to be used, due to the extra cost of pumping seawater.

Howard (1974) suggested that cages of 1.0 m depth, enclosing a volume of 20 m$^3$ would be capable of producing 1.5 tonnes of *S. maximus* over a two year production cycle. A unit to produce 100 tonnes/year could be contained within an area of one hectare. A production level of 25 kg/m$^3$ was achieved with *P. platessa* at an onshore site with heated water and a flow rate of 216 m$^3$/d. It was expected that with a flow rate of 500 m$^3$/d, a production level of over 40 kg/m$^3$ of *S. maximus* could be reached. Tanks of 50 m$^3$ were expected to produce 2 tonnes over a two year on-growing period.

Extensive culture methods using outdoor ponds with little environmental control, have been used for the production of *S. auratus* and *Solea solea* (sole) (Eisawy and Wassef,

1984; Jinadasa *et al.*, 1991). Good growth rates were achieved with both species, making this a possible option for the low cost growout of *R. tapirina*.

## 8.3. MARKETS

There is an existing domestic market for fresh gutted flounder of 500 g plus (recovery rate gilled and gutted = 97-98%). The hotel trade presently requires this product but can only acquire small quantities of frozen imports from New Zealand or fresh 350-420 g fish. The smaller fish are readily available and command much lower prices (D. Weller, pers. comm.).

A second market exists for a live product. *R. tapirina* are particularly suited to live transport as they are relatively easy to keep live for long periods with oxygen, but very little water. A market for live flounder exists in the Australian mainland capitals and in South East Asia, although the Japanese are already producing a flatfish species (*P. olivaceus*). The Japanese prefer left-eyed flatfish species rather than right-eyed such as *R. tapirina* and will pay almost twice as much for them. They will also pay more for fish with thick fillets (Anon, 1990).

In order to achieve the highest available market price for both live and gutted *R. tapirina* the size must be 500 g plus (D. Weller, pers. comm.). One of the concerns at present is whether this size can be achieved economically. There may also be a Japanese market for 80 g with ripening gonads or 'roe' but this requires further market research. Fish with approximately these specifications have been produced (Section 7.3.2.).

Gutted 500 g *R. tapirina* command a price of A$8-10/kg in the domestic market while those of 300-450 g only fetch around A$4-5/kg (D. Weller, pers. comm.). Prices are expected to rise by 10-15% in the next few years due to new regulations and quotas in the wild fishery. The market for live *R. tapirina* is both domestic and export. Values within Australia, particularly Sydney and Adelaide, have been quoted as being from A$15-24/kg. Outside Australia there is a large Asian market which includes Singapore and Hong Kong. This market has values quoted at around A$12/kg. Transport costs for live fish must be taken into account at this stage and they are as yet, unknown. However, the technology has already been developed in Japan for the transport of their cultured flatfish species. The cost of transporting fresh gutted *R. tapirina* from Hobart to Sydney is presently A$0.29/kg in 600 kg lots (D. Weller, pers. comm.).

At present most *R. tapirina* are sold either as fresh or frozen, gutted whole fish. In Europe flatfish species are sold in large quantities through supermarkets as fillets or

crumbed fillets. This opportunity exists in Australia. The opportunity may also exist for supplying fresh fillets to airline catering companies. However, this requires further investigation. It may be possible to produce 80 g fish containing roe for the Japanese market on a year-round basis if this market exists in volume. *R. tapirina* may also fit into the Japanese sushimi market. *P. olivaceus* is one of the most valuable Japanese fish products in the form of 'hirame' which is a raw sushimi product (Watanabe and Nomura, 1990).

Markets in Australia are quite substantial with at least 300 kg/week being sold in Hobart and at least 3 tonnes per week in Melbourne (D. Weller, pers. comm.). However, these figures do not take into account frozen imports and are only for the small fresh product. The present landings of *R. tapirina* in Tasmania are about 40 tonnes per annum with about 50 tonnes per annum landed in both the Victorian and South Australian fisheries (Kailola *et al.*, 1993). Landings in New Zealand are considerably higher at 1,000 tonnes per annum (Paul, 1986).

## 8.4. ADVANTAGES OF COMMERCIAL CULTURE

The present supply of flounder, in general, fluctuates considerably and is declining (Tasmanian Department of Sea Fisheries, statistics, 1993). A cultured product would have the advantage of regular and reliable supply. Alternatively, by supplying the product at times when it is not normally available, a higher price may be achieved. The main supply of wild fish appears to be available during the peak of the spawning season, between September and October.

*R. tapirina* is not being cultured anywhere else in the world although it occurs in New Zealand. New Zealand scientists have expressed an interest in its culture but are not presently in a position to start production, due to lack of technology. This would not take long to rectify if enough interest was shown. New Zealand presently exports considerable quantities of wild-caught flounder to Australia. These are normally frozen and a large proportion are *R. plebia* (sand flounder) rather than *R. tapirina*. The quality of *R. plebia* is considered to be inferior to that of *R. tapirina* due to a lower flesh to bone ratio. A number of other flatfish species are being produced in Europe and Japan and, more recently, Chile. These are mostly much larger species such as *S. maximus* and *H. hippoglossus* which fit into a different market niche and may therefore, not represent real competition at this stage. Within Australia there is considerable interest in flounder culture in all the southern states particularly South Australia (A. Heywood, pers. comm.).

Tasmania has the greatest abundance of *R. tapirina* in Australia and may therefore have the optimal climate. However, the other southern states all have warmer temperatures and may therefore achieve higher growth rates. On the other hand their summer temperatures may prove to be too high. The availability of farm sites for cage farming is probably better in Tasmania than in other states. However, this is not true of land-based sites. *R. tapirina* will be able to cope with warmer water conditions and shallower sites than are presently used for the production of *S. salar*. It is not possible to mix the two species on the same site due to production and disease problems.

*R. tapirina* require basic marine conditions, but a short-term experiment showed that they can tolerate salinities down to 15‰ with no loss of growth only slightly higher mortality. Their temperature range appears to be fairly broad as well, with optimal growth probably occurring between 18 and 22°C. They are a demersal species although they do rise off the bottom to feed in a culture situation. They do not require great depths of water and could probably be cultured successfully in tanks or cages of only 1-2 m depth as used for the culture of *S. maximus* and *S. solea* (Person-Le Ruyet, 1990). This means that the site required need be no more than 2-4 m deep depending on the water flow. Flatfish are particularly susceptible to damage by ultra violet light (Bullock, 1988) and so cages would probably need a shade cloth cover. As flatfish rest on the bottom, the movement of the cage base causes stress and interferes with feeding, which is the reason for questioning their suitability for cage culture. The netting may also cause abrasion leading to infection by opportunistic pathogens such as *Flexibacter maritimus*. The site must therefore be very sheltered. It is possible that a more extensive culture can be employed by fencing off areas of coast such as shallow bays. However, Tasmanian State Government policy may not allow this method to be used.

Land-based sites and hatcheries need to be in close proximity to the sea and electricity supplies for pumping water. They also require good water flows close to the shore so that effluent water is quickly dispersed and not pumped back through the farm. Some form of waste treatment such as a settlement pond may be required. Water quality at any marine site must be of very high quality; not subject to great fluctuations in turbidity, or salinity due to freshwater runoff from river systems, or pollution.

The species is very fecund and survival rate is reasonable so that only small numbers of broodstock need be collected, bearing in mind the need to prevent inbreeding. However, if inbreeding should occur, wild stock will always be available from which to increase the gene pool. Two stocks of *R. tapirina* were identified in Tasmania by Kurth (1957), on the basis of morphological characteristics. One occurs in Bass strait and the other on the East coast. The differences between the two in terms of culture

potential, have not been evaluated. The only identified differences between these stocks at present, are morphological.

As *R. tapirina* is a native species it is unlikely that local climatic aberrations will effect it. The main risks are those associated with the culture of any species, such as pollution, algal blooms, disease, predation by birds and marine mammals, and poaching. However, these will not present any greater risk to *R. tapirina* culture than to existing aquaculture industries in Tasmania.

## 8.5. DISEASE

A number of diseases have been recorded in cultured *R. tapirina* and these have been identified by the DPIF Fish Health Unit, Mount Pleasant Laboratories, Launceston. Major pathogens are:

1. ***Flexibacter maritimus.*** Both adults and juveniles are susceptible to outbreaks of *Flexibacter maritimus*, although this is always associated with stress or physical damage. The adults are particularly susceptible after stripping due to the damage and stress caused by this process. Juveniles during weaning are also susceptible due to loss of condition and deteriorating water quality. A flow-through system or sophisticated filtration may prevent this. Treatment of adults with acriflavine has been successful when applied directly to the affected area.

2. ***Trichodina* sp.** Adults and juveniles growing at the Bridport site have been affected by heavy infestations of *Trichodina* sp., possibly causing low level mortality. However, wild fish have also been shown to carry large populations of *Trichodina* sp. and it may therefore not represent a major threat. This is probably not a problem to hatchery or nursery facilities with adequate filtration of the intake water. Freshwater baths were unsuccessful in controlling the disease but formalin was effective (I. Cameron, pers. comm.). Person-Le Ruyet (1990) reported *Trichodina* infections in cultured *S. maximus* and recommended treatment with formalin and malachite green at a concentration of 100 ppm formalin and 0.5 ppm. malachite green for 1 hour.

3. ***Aeromonas salmonicida.*** The most potentially threatening disease identified in flounder cultured at Bridport was *A. salmonicida* which was isolated from a small percentage of specimens during 1993. The strain was different from that involved in diseases of commercial importance in other countries. However, the source of the disease is unknown. Salmonids reared in the same water were unaffected by the disease. Person-Le Ruyet (1990) reported *A. salmonicida*

infection in larval *S. maximus* and suggested that it was linked to deficiencies in the culture method leading to physiological malfunction and breakdown.

4. **Nutrition.** The nutrition of flounder is little understood at present. Nutritional deficiencies have been responsible for reductions in larval growth rates, increased deformities, frequency of malpigmentation, and cataracts of the eye. Vitamin C deficiency during weaning appears to be the cause of shortened opercula and lordosis in juvenile flounder weaned over long periods. The high fat content of trout pellets used to feed juveniles and adults appears to be the cause of fatty accumulation in the liver and possible reduced growth rates. This has also been shown to occur in *D. labrax* juveniles fed a dry diet containing 9% fat (Mosconi-Bac, 1987).

The major problem with the appearance of cultured *R. tapirina* at present, is the existence of many dark blotches on the undersurface of most fish. This may render them unsuitable for either the domestic or the export markets which require clean white undersurfaces to their fish. Many of the cultured fish also show signs of possible vitamin C deficiency in the form of shortened opercula and lordosis of the spine near the caudal peduncle. This reduces the external appearance of the fish and spinal deformities may interfere with processes such as filleting.

## 8.6. CONCLUSION AND FUTURE DIRECTIONS

The major findings of this study, and the main areas in which to concentrate future research efforts, are:

1. *R. tapirina* can be induced to spawn in captivity using interperitoneal injections of 0.5 ml/kg 'Ovaprim' with maturation occurring normally, even under artificial conditions of photoperiod and temperature. However, the fertilisation rates achieved were low. There is a need for further research into ovulatory cycles, effects of environmental cues, and nutrition of broodstock in order to achieve more consistent ovulations and higher fertilisation rates.

2. Optimal salinity for fertilisation of *R. tapirina* eggs lies in the range 35-45‰, with fertilisation unsuccessful in salinities below 15‰. The fertilisation method, wet or dry, has no effect on fertilisation rates. Hatch rates are unaffected by incubation salinities between 15 and 45‰, although an optimal incubation temperature range of 9-12°C is evident with a tolerance range of 6 -21°C. The eggs are buoyant at salinities above 27-28‰ and the time to 50% hatch ranges from 1,089 - 1,212 degree hours, depending on the incubation temperature. Eggs

can be transferred successfully from the incubation unit to a hatching unit at the tail-bud stage and it may therefore, be better to incubate the eggs in dedicated incubation systems, at high density, high salinity and low temperature, before transferral to the larval rearing unit.

3. The optimal temperature for the yolk absorption stage in *R. tapirina* is 15°C as this temperature results in the fastest growth rates and the maximum length at the time of complete yolk absorption. At this temperature first-feeding occurs at approximately 96 hours (day 4) post-hatch, between complete yolk-sac absorption and oil droplet absorption. The 'point of no return' occurs at day 6 post-hatch and mortality of unfed larvae occurs at day 8 post-hatch.

4. Rearing of *R. tapirina* larvae to metamorphosis can be carried out successfully in black hemispherical tanks, of 3, 25 and 200 l capacity, using recirculation systems and light intensities of 300-1,699 lux. The optimal photoperiod for maximum growth is 18-24 hours light, and the optimal temperature is 18-20°C. Salinities of 15‰ result in slightly increased mortality, compared to higher salinities, although there is a need for further research into the reasons for this as it appears to contrast with some of the other results quoted in the literature.

5. The marine rotifer (*Brachionus plicatilis*) is a suitable first-feed for *R. tapirina* larvae, even though the larvae are relatively small at first-feeding (2.98 mm with a gape height of 429 μm). Enriched instar II *Artemia* can be mixed with rotifers after day 9 post-hatch when the larval gape height reaches 690 μm and the larval length is 4.7 mm. The larvae commence feeding at the water surface but move to the tank base at approximately day 15 post-hatch, after which they go through three distinct colour changes to metamorphosis, which occurs at approximately day 30 post-hatch, with a larval length of 12.4 mm and weight of 25 mg, in the best batches. The stomach is fully formed by day 20 post-hatch. Enrichment with artificial diets compared to microalgae, improves the growth rates but sometimes appears to induce malpigmentation and cause higher mortality. Further research is required into enrichment diets, the development of the digestive enzyme system and the bacterial composition of the gut, in order to optimise the feeding regimes and larval nutrition for improved growth, survival and pigmentation.

6. Weaning to an artificial diet can be carried out from day 23 post-hatch using a 10 day changeover period during which the level of live feeds is reduced and that of artificial feeds is increased. The weaning diets trialed, and stocking densities used, had no effect on growth or survival rates. Changeover periods of

10 or 20 days had no significant effect on growth or survival but a weaning period of 5 days resulted in poor growth. Weaning earlier than day 50 post-hatch resulted in the highest survival rates. Larvae showing the highest growth rates prior to weaning, showed the best growth and survival rates during weaning, and therefore larval nutrition and environmental conditions prior to weaning, should be optimised. The use of microalgae as an enrichment during the first 15 days of larval rearing appeared to increase post-weaning growth and survival. The reasons for this require further research which should concentrate on the nutritional value of the microalgae and the bacterial composition of the larval gut.

7. Growth rates on dry diets are good and food conversion rates of approximately 1:1 can be achieved in the laboratory. However, there are problems with dark pigmentation on the blind side of the fish and a high incidence of spinal and opercula deformities. The causes of both these phenomena require further research. The prospects for growout are good but further research is required into the causes of early maturation, the type of holding structures required (e.g. tanks and cages), disease control and marketing.

The hatchery production of *R. tapirina* has significant commercial potential. The production of juveniles weaned onto available dry, commercial salmonid diets is already feasible on a large scale. However, the cost effectiveness of a complete culture method will depend on the results obtained from future research into nutrition, growout methods, disease and marketing. If a nutritionally complete diet can be designed, and a suitable growout method identified, then a market size of 500 g can possibly be attained in a 2 year period. If the present knowledge of potential disease problems can be extended to include methods of control, then the survival rates can be further increased. Finally, if high value markets can be obtained, then the higher returns may render the operation cost effective, and the commercial culture of the species viable.

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# APPENDIX 1

# GENERAL MATERIALS AND METHODS

## 1.1. WATER QUALITY MEASUREMENT

The University of Tasmania at Launceston has no direct access to seawater and consequently, relies on seawater from Bass Strait, collected at Weymouth on the North coast of Tasmania. This seawater is brought in regularly by road in a converted stainless steel milk-tanker and stored in two 22,000 l concrete tanks. The seawater is continuously passed through a 50 µm sand-filter and pumped around the Key Centre for Teaching and Research in Aquaculture along 50 mm PVC pipes. The marine fish hatchery has access to an outlet from this main piping system. The system is regularly flushed through with Hypochlorite solution and rinsed with freshwater.

Seawater for use in the larval rearing tanks, was stored in aerated, 300 l plastic barrels until required and this allowed it to acclimatise to room temperature, which was normally maintained at a constant 15°C. No further treatment was carried out prior to use.

Salinity was measured with a refractometer S-10, Atago Co. Ltd., Japan, or a Yeo-cal model 602, Mk. II, salinity/temperature bridge. The salinity in the larval tanks was maintained at 35±2‰ and was diluted with fresh water acclimated to temperature, when it rose above 35‰ due to evaporation.

Temperature was measured with a mercury thermometer calibrated against an electric probe.

Ammonia ($NH_3$) and nitrite ($NO_2$) were measured using a DR/2000 Hach direct reading spectrophotometer. The concentration of $NH_3$ never exceeded 0.1 mg/L and that of $NO_2$ never exceeded 0.04 mg/L.

pH measurements were made using an Activon, Model 209 pH/mV meter. The pH was maintained at around 7.8.

Dissolved oxygen (D. O.) was measured with a Yeo-Cal, Model 603, oxygen meter. D.O. was maintained above 5 mg/L but did not fall below 7 during the live feeding stages of rearing. Weaning was more problematic as suspended solids and organic

matter built up in the water column increasing the biological oxygen demand (BOD). However, it was never the cause of direct mortality.

## 1.2. LIVE FOOD PRODUCTION

Algae was cultured in 200 l bags using $F_2$ medium (Guillard and Ryther, 1962). Rotifers (*Brachionus plicatilis* ) were fed on a mixture of algae (*Tetraselmis chui, Pavlova lutheri* and *Isochrisis* sp. [Tahitian clone]) and yeast and reared in 500 l cylindrical white fibreglass tanks at a temperature of 22-24°C. Densities were maintained at around 100-150/ml in seawater of 35‰. Rotifers were rinsed at harvest with clean seawater and enriched for 18-24 hours with algae (*Pavlova lutheri* and *Isochrisis* sp. [Tahitian clone]) except in the experiments described in section 5.4.3. Brine shrimp (*Artemia* sp.) from the Great Salt Lake (mainly Olympia brand), were either decapsulated using the method described by Sorgeloos *et al.* (1977) or more usually, hatched in dilute seawater at 22-24°C, without decapsulation and the hatched cysts removed from the surface of the hatching container using a siphon tube. Only instar II nauplii were used for feeding flounder larvae, and these were enriched with the microalgae (*Pavlova lutheri* and *Isochrisis* sp. (Tahitian clone)), in seawater of 35‰ at 22-24°C, for 18-24 hours, except in the experiments described in section 5.4.3. In 1993 Frippak was used as the normal enrichment for both rotifers and *Artemia*, due to the promising results obtained in previous years. White buckets of 20 l capacity were used for enrichment and light aeration was supplied by an airline, weighted to rest on the bottom of the bucket. Both rotifers and *Artemia* were rinsed in seawater or freshwater respectively, before introducing to the larval rearing tanks. Live feeds were added to the rearing tanks twice daily, at 9 am and 5 pm, except during weaning (Chapter 6). The numbers of live organisms to add to the rearing tanks were calculated to give the required density over the two feeds. No counts of feed densities were carried out in the rearing tanks due to the uneven distribution of animals within the water column and potential damage to the fish larvae by physical mixing. Counts of densities in the rotifer and *Artemia* rearing tanks were carried out by removing a sample from the tank after heavy mixing by aeration and counting 3 samples of 1 ml each under a dissecting microscope. Total numbers could then be estimated by extrapolation.

REFERENCES

Guillard, R.R.L. and Ryther, J.H., 1962. Studies on marine planktonic diatoms. 1. *Cyclotella nana* Hustedt and *Dotonula confervacae* (Cleve) Gran. Can. J. Microbiol., 8: 229-239.

Sorgeloos, P., Bossyut, E., Lavina, E., Baeza-Mesa, M. and Persoone, G., 1977. Decapsulation of *Artemia* cysts: a simple technique for the improvement of the use of brine shrimp in aquaculture. Aquaculture, 12: 311-315.

## 1.3. SYSTEMS USED FOR INCUBATION AND MASS-REARING

The larval rearing unit used for a preliminary study of larval rearing in the first year (1990), consisted of four 25 l black hemispherical fibreglass tanks fitted in line, in a recirculating seawater system (Fig. 1.). The inlets were of 13 mm black PVC, fitted with an adjustable tap and entering the tank at bottom centre. Water flow was set at approximately 6 l/h. Outlets were at top centre and consisted of 15 mm perforated, white PVC pipes with an aquarium sponge filter fitted to the end. Water leaving each tank entered a 50 mm PVC drainpipe via a flexible, removable outlet pipe. The drainpipe flowed into the biofilter via numerous 7 mm holes. The biofilter consisted of oyster shellgrit, contained in a fishbox and suspended over a 450 l reservoir. Water was pumped directly from the reservoir back to the tanks, by an Onga FP10 submersible pump.

The larval rearing unit was kept in an air-conditioned room maintained at a constant temperature of 15°C. A 24 hour light regime was implemented using a fluorescent light suspended approximately 0.5 m above the tanks. Regular water changes were carried out and water quality was monitored throughout the period. A 5 mm siphon tube was used to clean any solid debris from the tank bottom on a daily basis. This system was used successfully to culture *R. tapirina*. However, the results are not described in this document.

In the second year of the study (1991), an insulated hatchery building was constructed specifically for marine fish production. The building contained an air conditioner which was set at 15°C and fluorescent lighting with photoperiod control. Four sets of 160 l black hemispherical fibreglass tanks were constructed, each containing 3 tanks with a 200 l reservoir and a trickle filter containing artificial substrate (bioballs) set above a submerged filter containing shellgrit. These systems were used for incubation of eggs and mass rearing of larvae for experimental work, although some assessment of behaviour, development and the effect of enrichment diets, was carried out without replication (Section 5.2.).

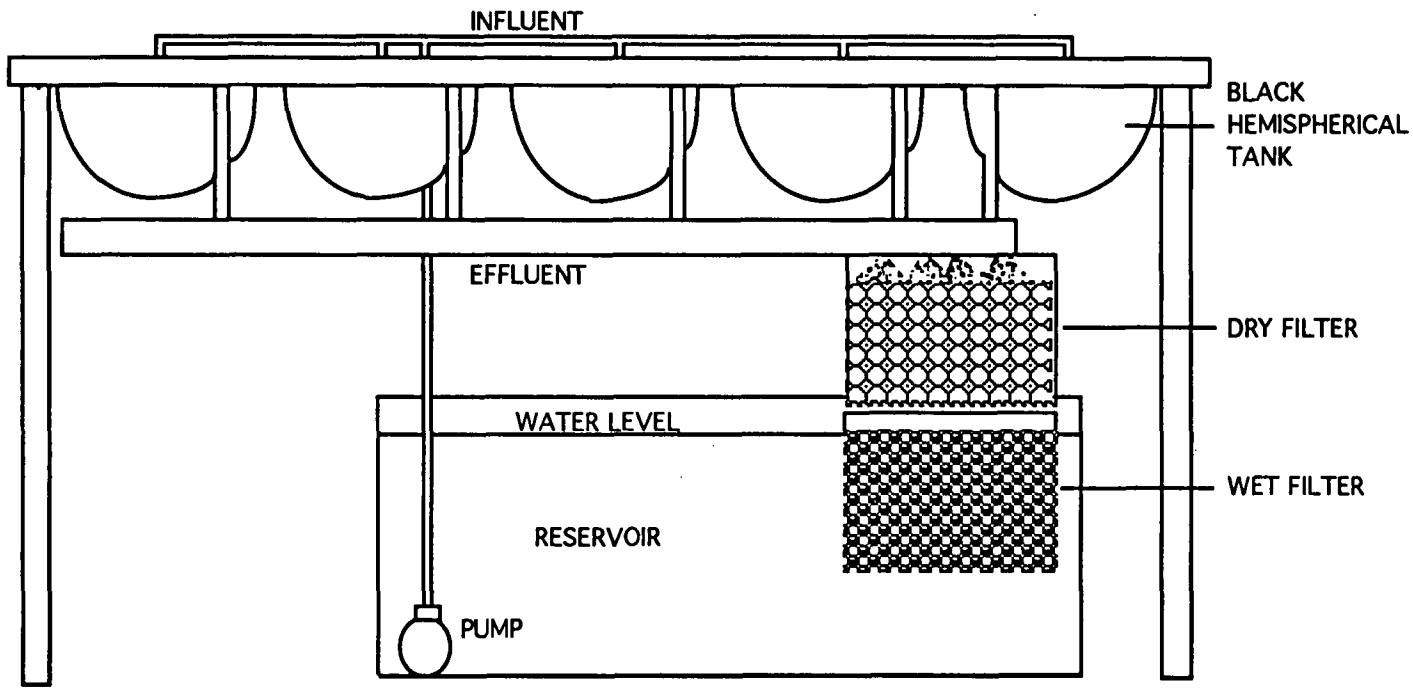


Fig. 1. Diagram of the recirculating system used for incubation, larval-rearing and weaning. Tanks were constructed of black fibreglass and contained 25 l of water. Each system contained either 4 or 9 tanks. Filtration equipment was the same for the 160 l systems described above.

## 1.4. DATA COLLECTION AND ANALYSIS

All fish were anaesthetised with benzocaine prior to analysis. Weights of larvae were measured on a Mettler AE 260 balance (± 0.5 mg) and juveniles were weighed on a Mettler PM 4800 balance (± 5 mg). Measurements of larvae were taken using an eyepiece micrometer on a low power binocular microscope (± 0.033 or 0.5 mm) and juveniles were measured using callipers (± 0.05 mm). Measurements of juveniles were hampered by the fact that *R. tapirina* does not have a fork to the tail and so total length had to be used and some fish had foreshortened caudal fins due to abrasion and fin-biting. This led to occasional measurements that were unrealistically short. However the occurrence of this phenomenon was considered to be uncommon and therefore did not have a significant effect on the results. It did however, effect some of the calculations of condition factor which were abandoned as a measure of condition in weaning experiments where fin-biting was particularly common while the fish were partially starved during the process.

Sample sizes are stated in the detailed method sections. In general, juvenile samples were kept to a minimum of 20 fish with 30 fish measured for final samples and 50 for initial samples.

All the data collected at the termination of experiments, were subjected to a one-way (or two-way, section 4.3.2. (i)) analysis of variance (ANOVA) using Fisher's test for least significant differences (LSD) at the 95, or 99% confidence level. Homogeneity of variance was tested using Bartlett's test and normality of distribution was tested using a Shapiro-Wilk W test on the residuals of the replicate means or on the individual samples where there was no replication. The JMP® 2.0 statistical package for Mackintosh computers was used for all statistical analyses.

## 1.5. STAGING USED BY CRAWFORD (1984b)

### 1.5.1. External appearance of the gonads

**Table 1.5.1. Maturity stages recognised amongst undissected *R. tapirina*. (Crawford 1984b).**

| Maturity Stage | | Description |
|---|---|---|
| Ovaries | | |
| A | Immature-Resting | Ovaries small, posterior extension approximately half way to caudal peduncle |
| B | Developing-Partially Spent | Ovaries larger, firm, posterior extension approximately three quarters way to caudal peduncle |
| C | Mature | Ovaries enlarged and distended, bulging above body musculature, posterior extension almost to caudal peduncle |
| D | Spent | Ovary thin and flabby, still extends almost to caudal peduncle |
| Testes | | |
| A | Immature-Spent-Resting | No milt exuded under firm pressure |
| B | Running Ripe | Milt exuded under gentle pressure |

### 1.5.2. Larval stages

**1.5.2. Stages recognised during larval development (Ryland, 1966; Crawford, 1986)**

| Stage | Description |
|---|---|
| 1 | Yolk sac present |
| 2 | Yolk resorbed, notochord straight |
| 3/4 | Flexion of notochord and migration of left eye |
| 5 | Eye on or over edge of head |
| M | Metamorphosis |

## 1.6. EXPERIMENTAL DESIGN CONSIDERATIONS FOR EGG AND YOLK SAC EXPERIMENTS

A number of potentially limiting factors were identified, during preliminary trials, and these had to be rectified before a final design was implemented. These were:

### Egg experiments

*a. the unequal buoyancy of eggs*

Eggs at 25‰ and 15‰ sank to the bottom of the pots while eggs at 35‰ and 45‰ floated at the water surface. This meant that at the lower salinities the live eggs were in direct contact with dead eggs which also sank and were therefore prone to bacterial attack. It was not possible to remove dead eggs as this would have meant disturbing the live eggs which might have affected the results. Spectorova *et al.* (1974) also noted this as being a problem with the eggs of *S. maximus maeoticus*.

*b. water quality deterioration*

It was not possible to carry out water exchanges as removing water from the beakers would have disturbed any floating eggs in the high salinity treatments but not those that sank in the low salinity treatments. The unequal levels of disturbance might have affected the results. The breakdown of dead eggs was therefore a potential cause of reduced water quality in terms of reduced oxygen levels and increased ammonia and bacteria levels. Evaporation of water from the beakers was also a problem resulting in fluctuating salinities. Aeration could not be used due to the small size of the beakers and the delicate nature of the eggs.

*c. temperature/salinity shock during initial transfer*

As the broodstock were held in seawater of 35‰ and 12°C, many of the experimental batches of eggs had to be transferred to considerably different conditions of temperature and salinity in the experimental system. It was not acceptable to fertilise the eggs in water of the experimental temperature and salinity as the fertilisation rates can be affected by these conditions (section 2.3.3. (iii)). Also, a proportion of unfertilised eggs and excess sperm would have been present in the experimental beakers, resulting in greater biological oxygen demand and bacterial numbers, as they decayed.

*d. distribution and counting of eggs.*

Distributing an equal and measured, number of eggs to each beaker was important as too many eggs resulted in water quality deterioration and too few eggs resulted in too few survivors to allow statistical analyses of the results. Counting eggs individually caused physical damage and took too long to perform.

*Solutions.*

The final experimental design was similar to that used by Kuhlmann and Quantz (1980) for the eggs of *S. maximus*. Low numbers of eggs were used to prevent water quality deteriorations and the need for water exchanges. This unfortunately, prevented collection of data on the length of the larvae at hatching as there were too few hatched larvae in each beaker to allow meaningful statistical analyses. The use of artificial seawater reduced bacterial levels and lids placed loosely on the beakers prevented evaporation.

The eggs were fertilised in seawater of 15°C and 35‰ (using the general method described in section 2.2.3. (i)) so that unfertilised and dead eggs and excess sperm could be removed prior to distribution to the experimental beakers. Fertilised eggs were pipetted from the fertilisation beaker and the quantity of eggs was measured by volume, in the pipette. An equal volume was placed into each experimental beaker, within 1 hour of fertilisation (prior to 1st cleavage) and therefore, before the cells had commenced differentiation, so that normal development would not be affected by the temperature/salinity shocks incurred.

**(ii) Yolk sac experiments**

*a. water quality deterioration.*

In earlier trials the dead larvae decayed rapidly, particularly at the higher temperatures and caused oxygen depletion and increased ammonia and bacterial levels. In the final experiment water filtered to 1 μm was used and a 25‰ water change was carried out every second day.

*b. developmental stage at transfer.*

Transfer at the pre-hatching stage resulted in variations in the hatch rate due to the effect of temperature. In the final experiment the larvae were transferred to the

experimental beakers just after hatching and the water was allowed to acclimate to temperature over several hours after the transfer.

*c. identifying the point of complete yolk absorption.*

The larvae had to be sampled as close to the time of complete yolk absorption as possible. It was only possible to determine accurately when this had occurred by microscopic examination of the larvae. Therefore, when it was judged that the yolk and oil droplet had been fully absorbed at a particular temperature, a single larva was removed from one beaker and observed under a dissecting microscope to provide confirmation. This was found to be a reliable method as all the larvae at each temperature, completed yolk absorption at approximately the same time.

## APPENDIX 2.

## REPLICATE MEANS FROM CHAPTER 2

### 2.1. METHODS OF FERTILISATION

| Method | Wet | | Wet eggs | | Wet sperm | | Dry | |
|---|---|---|---|---|---|---|---|---|
| Total no of eggs | 210 | 234 | 170 | 102 | 163 | 97 | 59 | 103 |
| Fertilisation (%) | 52 | 59 | 55 | 79 | 50 | 59 | 63 | 50 |

### 2.2. THE EFFECT OF SALINITY ON THE FERTILISATION RATE

| Salinity (‰) | Fertilisation (%) | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| 45 | 91 | 89 | 91 |
| 35 | 93 | 923 | 94 |
| 25 | 81 | 71 | 76 |
| 15 | 22 | 10 | 12 |
| 5 | 0 | 0 | 0 |

# APPENDIX 3.

# REPLICATE MEANS FROM CHAPTER 3

## 3.1. THE EFFECT OF SALINITY AND TEMPERATURE ON EGGS

| 18°C | Hatch rate (%) | | |
|---|---|---|---|
| Salinity (‰) | 1 | 2 | 3 |
| 45 | 3 | 0 | 0 |
| 35 | 13 | 5 | 0 |
| 25 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 |

| 15°C | Hatch rate (%) | | |
|---|---|---|---|
| Salinity (‰) | 1 | 2 | 3 |
| 45 | 43 | 445 | 49 |
| 35 | 30 | 31 | 33 |
| 25 | 33 | 47 | 44 |
| 15 | 33 | 27 | 39 |

| 12°C | Hatch rate (%) | | |
|---|---|---|---|
| Salinity (‰) | 1 | 2 | 3 |
| 45 | 50 | 54 | 47 |
| 35 | 52 | 66 | 0* |
| 25 | 50 | 61 | 41 |
| 15 | 34 | 41 | 38 |

| 9°C | Hatch rate (%) | | |
|---|---|---|---|
| Salinity (‰) | 1 | 2 | 3 |
| 45 | 39 | 38 | 43 |
| 35 | 56 | 38 | 0* |
| 25 | 423 | 45 | 77 |
| 15 | 43 | 53 | 54 |

* omitted from analysis as mortality was due to contamination of pots used for 35‰ replicates and these data become outliers.

## 3.2. THE EFFECT OF TEMPERATURE ON YOLK-SAC LARVAE

| Temperature | Final length (mm) | | | Survival (%) | | | Growth rate (mm/d) | | | Deformities (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (°C) | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 18 | 2.71 | 2.67 | 2.69 | 75 | 91 | 89 | 0.13 | 0.12 | 0.12 | 5 | 0 | 10 |
| 16.5 | 2.73 | 2.7 | 2.76 | 80 | 93 | 93 | 0.13 | 0.12 | 0.14 | 5 | 0 | 0 |
| 15 | 2.77 | 2.76 | 2.76 | 45 | 81 | 71 | 0.11 | 0.11 | 0.11 | 0 | 5 | 10 |
| 12 | 2.78 | 2.73 | 2.78 | 80 | 22 | 10 | 0.12 | 0.10 | 0.12 | 0 | 10 | 5 |
| 9 | 2.87 | 2.73 | 2.81 | 85 | 0 | 0 | 0.09 | 0.07 | 0.08 | 10 | 15 | 15 |

### 3.3. TIME OF FIRST-FEEDING

| Day first fed | Final length (mm) | | | Survival (%) | | |
|---|---|---|---|---|---|---|
| (d) | 1 | 2 | 3 | 1 | 2 | 3 |
| 3 | 4.71 | 4.90 | 4.58 | 78 | 50 | 66 |
| 4 | 4.67 | 4.68 | 4.59 | 86 | 66 | 52 |
| 5 | 4.11 | 4.2 | 4.14 | 28 | 64 | 52 |

### 3.4. THE EFFECT OF PHYSICAL DISTURBANCE

| | Hatch rate (%) | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Disturbed | 56 | 81 | 76 |
| Undisturbed | 79 | 83 | 59 |

## APPENDIX 4.

## REPLICATE MEANS FROM CHAPTER 4

### 4.1. PHOTOPERIOD

| Daylength (h) | Length at d 10 (mm) | | | Length at d 20 (mm) | | | Survival day 10-20 (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 24 | 4.02 | 4.14 | 3.96 | 6.48 | 6.7 | 6.32 | 69 | 73 | 78 |
| 18 | 3.82 | 3.78 | 3.82 | 6.56 | 6.55 | 6.17 | 73 | 86 | 88 |
| 9: 3+9:3 | 3.74 | 3.84 | 4.08 | 0 | 6.56 | 6.50 | 0 | 77 | 62 |
| 12 | 3.65 | 3.63 | 3.72 | 6.11 | 6.0 | 5.91 | 68 | 98 | 35 |
| 6 | 2.92 | 3.13 | 2.92 | 4.16 | 4.37 | 4.37 | 83 | 33 | 38 |
| 0 | 0 | 0 | 2.96 | 0 | 0 | 0 | 0 | 0 | 0 |

### 4.2. TEMPERATURE

| Temperature (°C) | Final Length (mm) | | | Final Weight (g) | | | Survival (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 20 | 14.43 | 13.90 | 13.63 | 0.043 | 0.038 | 0.038 | 80 | 88 | 86 |
| 19 | 13.73 | 14.44 | 14.01 | 0.038 | 0.044 | 0.038 | 90 | 80 | 88 |
| 18 | 13.45 | 13.56 | 14.09 | 0.036 | 0.037 | 0.039 | 96 | 76 | 96 |
| 17 | 11.67 | 13.09 | 11.68 | 0.024 | 0.034 | 0.024 | 96 | 92 | 90 |
| 16 | 12.36 | 12.91 | 12.79 | 0.030 | 0.037 | 0.032 | 70 | 60 | 90 |
| 15 | 10.9 | 11.25 | 11.58 | 0.021 | 0.023 | 0.024 | 88 | 86 | 82 |

### 4.3. SALINITY

| Salinity (‰) | Final Length (mm) | | | Survival (%) | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 35 | 15.0 | 14.57 | 15.11 | 100 | 100 | 100 |
| 25 | 14.75 | 14.67 | 14.58 | 88 | 96 | 100 |
| 15 | 15.28 | 14.39 | 14.72 | 90 | 94 | 90 |

# APPENDIX 5.

# REPLICATE MEANS FROM CHAPTER 5

## 5.1. ENRICHMENT (TRIAL 1.)

| Enrichment | Final Length (mm) | | | Final Weight (g) | | | Survival (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Frippak | 15.74 | 16.25 | 16.02 | 0.054 | 0.062 | 0.052 | 68 | 60 | 60 |
| Diet A | 14.51 | 14.94 | 14.3 | 0.044 | 0.051 | 0.043 | 67 | 68 | 86 |
| Microalgae | 14.61 | 14.08 | 14.16 | 0.042 | 0.037 | 0.04 | 84 | 90 | 67 |

## 5.2. ENRICHMENT (TRIAL 4)

| Enrichment Diet | Malpigmentation rate (%) | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Algae/Frippak | 0.7 | 0.3 | 1 |
| Frippak | 3.7 | 3.7 | 3.7 |
| Nutri-Pack | 2 | 4.3 | 2.3 |

# APPENDIX 6.

# REPLICATE MEANS FROM CHAPTER 6

## 6.1. WEANING DIETS

| Artificial diet | Final Length (mm) | | | Final Weight (g) | | | Survival (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Lansy | 20.4 | 20.99 | 18.77 | 0.138 | 0.139 | 0.119 | 14 | 13 | 21 |
| Biodiet/Skretting | 19.9 | 21.45 | 19.82 | 0.138 | 0.176 | 0.126 | 21 | 12 | 13 |
| Sevbar | 21.67 | 21.08 | 24.04 | 0.156 | 0.138 | 0.161 | 8 | 5 | 9 |

## 6.2. STOCKING DENSITY

| Stocking density | Final Length (mm) | | | Final Weight (g) | | | Survival (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| (No./l) | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 20 | 22.16 | 24.34 | 23.82 | 0.172 | 0.234 | 0.259 | 60 | 34 | 42 |
| 10 | 21.43 | 22.63 | 23.95 | 0.187 | 0.198 | 0.236 | 50 | 45 | 46 |
| 5 | 22.47 | 21.62 | 22.66 | 0.182 | 0.176 | 0.185 | 50 | 42 | 39 |

## 6.3. LENGTH OF CHANGEOVER

| Changeover | Final Length (mm) | | | Final Weight (g) | | | Survival (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| (d) | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 20 | 17.28 | 17.67 | 18.77 | 0.091 | 0.098 | 0.124 | 61 | 73 | 73 |
| 10 | 16.89 | 15.42 | 15.58 | 0.085 | 0.066 | 0.063 | 73 | 79 | 70 |
| 5 | 13.52 | 13.71 | 15.24 | 0.045 | 0.043 | 0.064 | 63 | 59 | 77 |

## 6.4. AGE AT WEANING

| Age at weaning | Final Length (mm) | | | Final Weight (g) | | | Survival (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| (d) | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 23* | 15.16 | 14.69 | 14.97 | 0.048 | 0.042 | 0.043 | 82 | 75 | 90 |
| 42 | 26.78 | 27.32 | 25.95 | 0.307 | 0.3 | 0.277 | 54 | 66 | 55 |
| 50 | 26.17 | 28.02 | 0 | 0.253 | 0.314 | 0 | 44 | 58 | 0 |
| 58 | 26.68 | 27.99 | 27.64 | 0.27 | 0.313 | 0.3 | 39 | 42 | 33 |

* from experiment 6.3.4 (b)

## 6.5. CONDITION PRIOR TO WEANING (TRIAL 1)

| Enrichment | Final Length (mm) | | | Final Weight (g) | | | Survival (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Algae | 20.11 | 20.81 | 20.35 | 0.123 | 0.145 | 0.126 | 44 | 51 | 48 |
| Algae /Frippak | 26.06 | 25.45 | 23.75 | 0.296 | 0.281 | 0.22 | 62 | 65 | 56 |
| Frippak | 22.8 | 23.44 | 23.45 | 0.221 | 0.196 | 0.203 | 53 | 61 | 60 |

## 6.6. CONDITION PRIOR TO WEANING (TRIAL 2)

| Enrichment | Final Length (mm) | | | Final Weight (g) | | | Survival (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Algae/Frippak | 13.78 | 13.57 | 13.36 | 0.045 | 0.039 | 0.042 | 51 | 31 | 25 |
| Frippak | 13.39 | 13.79 | 13.62 | 0.043 | 0.038 | 0.037 | 41 | 43 | 25 |
| Nutri-Pack | 13.8 | 13.4 | 13.35 | 0.042 | 0.044 | 0.040 | 14 | 41 | 43 |

## 6.7. FEED RATE PRIOR TO WEANING

| Feed Rate | Final Length (mm) | | | Final Weight (g) | | | Survival (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| (*Artemia*/ml) | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 10 | 20.77 | 26.6 | 23.03 | 0.154 | 0.332 | 0.199 | 44 | 36. | 39 |
| 5 | 20.28 | 20.44 | 23 | 0.157 | 0.153 | 0.237 | 39 | 43 | 44 |
| 1 | 17.42 | 19.76 | 19.08 | 0.092 | 0.125 | 0.127 | 43 | 54 | 54 |